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PRINCIPAL INVESTIGATOR: **Ceshi Chen**

CONTRACTING ORGANIZATION: **Albany Medical College, Albany, NY, 12208**

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14. ABSTRACT E3 ubiquitin ligase gene WWP1 is a candidate oncogene at 8q21 in human prostate because of gene amplification and over-expression. The purpose of the research is to understand the role and mechanism of WWP1 in prostate cancer development. In a four-year period, we studied functions of WWP1 and its related proteins (KLF5, PMEPA1, RNF11, Erbb4, p63 etc) in prostate and breast cancers. We demonstrated that WWP1 regulates cell proliferation, apoptosis, and migration through ubiquitinating RNF11, KLF5, Erbb4, and p63. The WWP1 interacting protein, PMEPA1, promotes AR-negative prostate cancer cell cycle through suppressing p21 expression. We published 9 research papers, 3 review articles, and 21 abstracts based on this award in the past four years.					
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Introduction

E3 ubiquitin ligase gene WWP1 is a candidate oncogene at 8q21 in human prostate and breast cancer (1) because of gene amplification and over-expression. Transient WWP1 overexpression promotes and transiently WWP1 knockdown inhibits prostate and breast cell proliferation. Accumulating evidence suggests that WWP1 negatively regulates the TGF β signaling pathway (2-4). WWP1 inhibits TGF β -induced transcriptional activities as well as PAI-1 and Jun B expression (2, 3). In addition, overexpression of WWP1 in MDCK cells reduces the TGF β -induced growth inhibition (2). Among eight Smads, WWP1 can strongly interact with Smad2, 3, 6, 7 proteins, weakly with Smad1 and 5, but not with Smad4 and Smad8, which do not contain a PY motif (2, 3). WWP1 directly targets Smad2 for ubiquitination and degradation in the presence of TGIF (2, 3). Two independent studies suggest that WWP1 targets T β R1 for ubiquitin-mediated degradation through the Samd7 adaptor (2, 3). Morén et al., reported that WWP1 uses Samd7 to induce Smad4 ubiquitination and degradation (5). Thus, WWP1 inhibits the TGF- β signaling pathway directly or indirectly by targeting the T β R1, Smad2, and/or Smad4 proteins for degradation. Previously, we demonstrated that WWP1 targets KLF5 (6) for ubiquitin mediated degradation.

The purpose of the research is to understand the role and mechanism of WWP1 in prostate cancer development.

Body

Task 1. To test the hypothesis that over-expression of WWP1 promotes and maintains human prostate carcinogenesis:

1. Prepare lentivirus expressing the WWP1 or LacZ gene, infect PZ-HPV7 and 22Rv1 prostate cell lines and isolate stable clones;
2. Prepare lentivirus expressing the WWP1 or Luc shRNA^{mic}, infect PC-3 and C4-2 prostate cell lines and isolate stable clones;
3. Compare the growth rate, cell cycle, motility and invasion, as well as TGF-beta, androgen, and drug response for cell models established above;

Using a lentiviral system, we firstly overexpressed WWP1 and WWP1m in RWPE1 (similar to PZ-HPV7) immortalized prostate and 22Rv1 prostate cancer cell lines. We first obtained one WWP1 (#1) and one WWP1m (#12) overexpressing RWPE1 clone respectively (**Fig. 1A**). We failed to get a LacZ control overexpressing RWPE1 clone. We examined the cell proliferation of these cells by DNA synthesis. As shown in **Fig. 1B**, WT WWP1 slightly increases the ³H-thymidine incorporation and WWP1m significantly suppressed the DNA synthesis compared to the parental RWPE1 prostate cells. However, these clones are unstable as the WWP1 expression was lost after two passages.

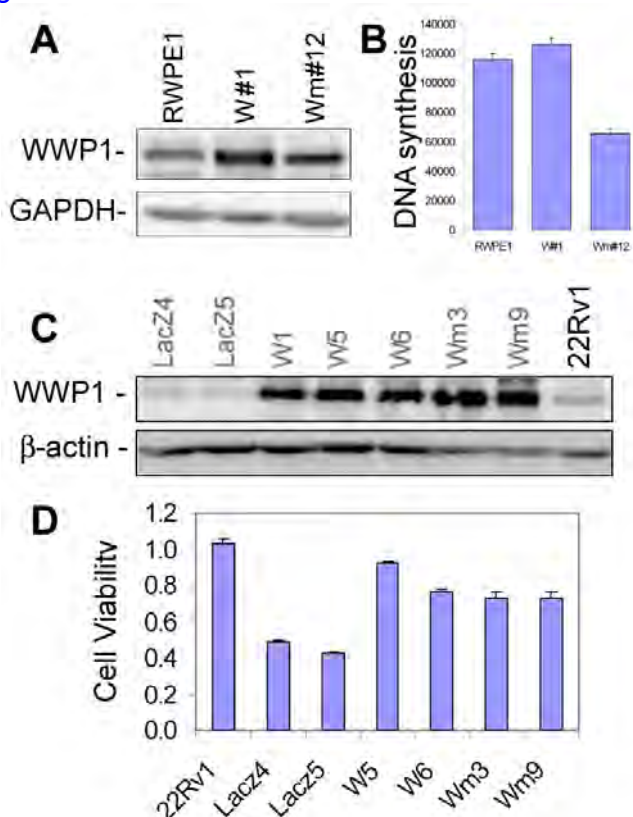


Fig. 1. Overexpression of WWP1 in prostate cells. **A.** WWP1 and WWP1C890A are overexpressed in RWPE1 “stable” clones compared to the parental cells. **B.** WWP1m decreases the DNA synthesis in RWPE1. **C.** WWP1 and WWP1C890A are overexpressed in 22Rv1 stable clones compared to the parental cells and LacZ clones. **D.** Both WWP1 and WWP1C890A increase the cell viability compared to the LacZ control, as determined by the SRB assay.

In the 22Rv1 prostate cancer cell line, we indeed obtained multiple WWP1 and WWP1m overexpressing clones (**Fig. 1C**). These clones are stable as WWP1 can be detected after at least 7 passages. We compared the cell proliferation/apoptosis index by the SRB assay. As shown in **Fig. 1D**, both WWP1 and WWP1m increase cell viability compared to the LacZ control. There is no significant difference between WWP1 and WWP1m. Anchorage independent growth of these clones showed a similar trend (data

not shown). In conclusion, WWP1 overexpression may promote prostate cell growth in E3 ligase independent manner.

We also successfully expressed WWP1 using lentivirus in MCF10A and MDA-MB-231 cell lines and found that WWP1 promotes cell proliferation in an E3 ligase activity independent manner (**Fig. 2**).

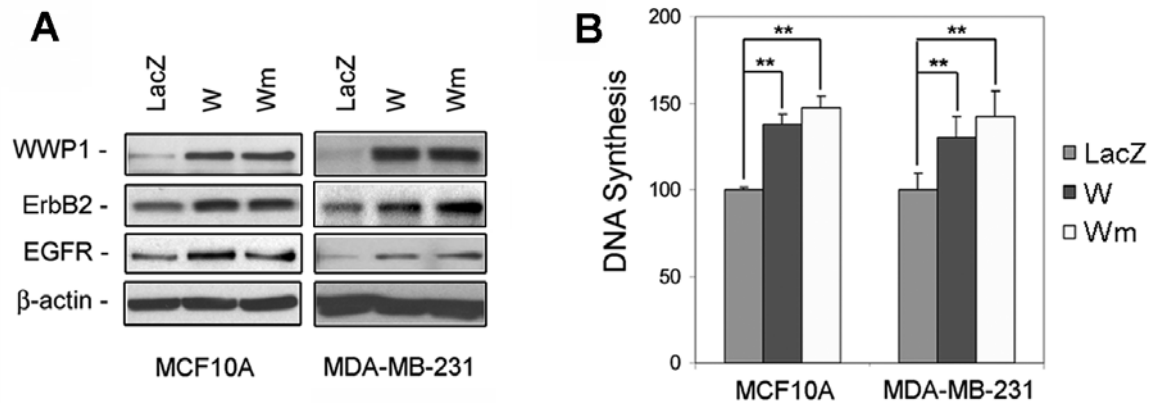


Figure 2. WWP1 regulates ErbB2/ EGFR and cell proliferation/survival in breast cell lines. **A.** WWP1 over-expression in MCF10A and MDA-MB-231 increases the protein levels of ErbB2 and EGFR in a ligase activity independent manner, as determined by Western blot. **B.** WWP1 significantly promotes DNA synthesis in MCF10A and MDA-MB-231, as determined by 3H thymidine incorporation. The LacZ control was defined to be 100. **, P<0.01 (t-test). W is the WT human WWP1, and Wm is the catalytic inactive human WWP1C890A. The cell populations with a low passage number (<3) were used in this study.

Additionally, we obtained WWP1 and catalytic inactive WWP1C890A (WWP1m) overexpressing MCF7 clones and found that the WT WWP1 can rescue the WWP1 ablation and TRAIL (25ng/ml) induced apoptosis (**Fig. 3A-B**). However, WWP1m functions as a dominant negative mutant to promote apoptosis induced by the WWP1 siRNA and TRAIL. Consistently, WWP1 promotes, but WWP1m inhibits anchorage independent growth (**Fig. 3C**).

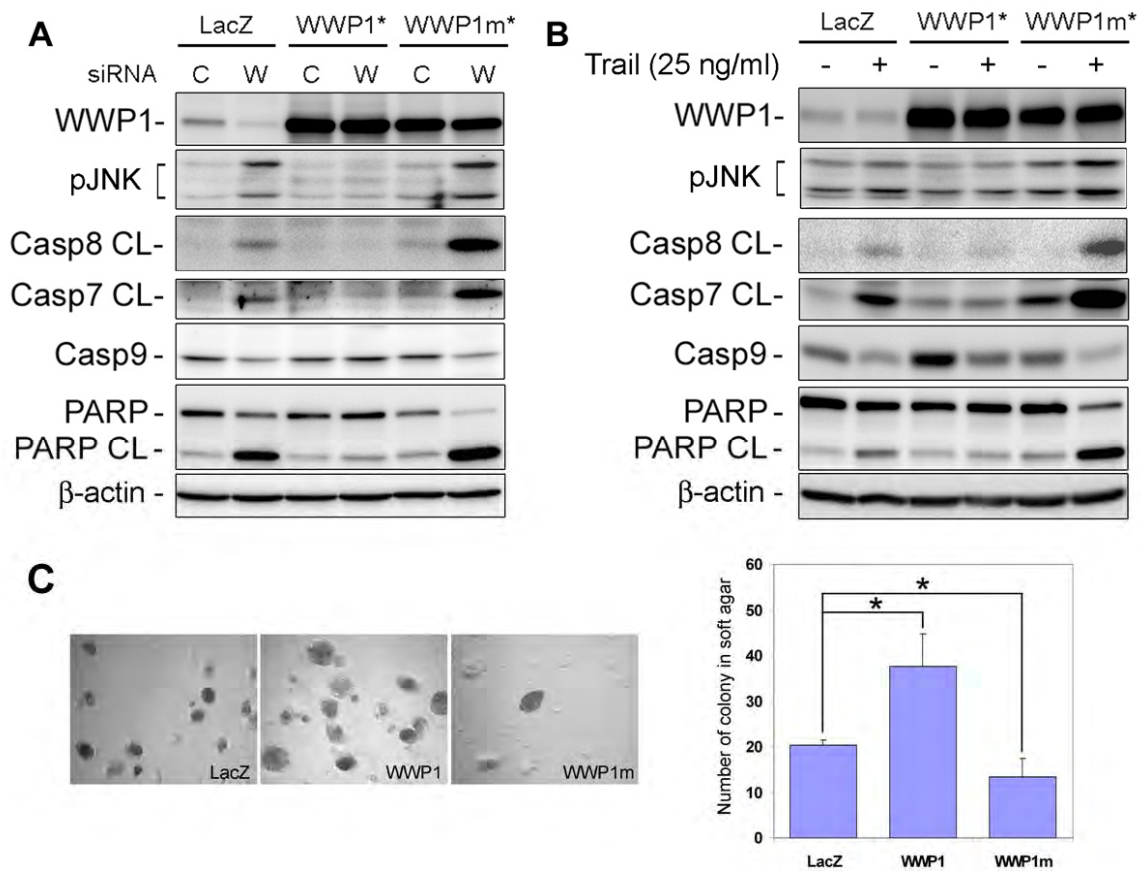


Fig. 3. Overexpression of WT WWP1 rescues the WWP1 siRNA induced apoptosis and causes resistance to TRAIL. **A.** WWP1 siRNA induces apoptosis (cleavage of Caspase 8, 7, 9 and PARP) in LacZ overexpressing MCF7 cells compared to the Lucsi control. WT WWP1 overexpressing MCF7 cells are resistant to the WWP1 siRNA. WWP1m overexpressing MCF7 cells become more sensitive to the WWP1 siRNA. **B.** TRAIL induces apoptosis (cleavage of Caspase 8, 7, 9 and PARP) in LacZ overexpressing MCF7 cells. WT WWP1 overexpressing MCF7 cells are resistant to TRAIL. WWP1m overexpressing MCF7 cells become more sensitive to TRAIL. **C.** WT WWP1 increases, but WWP1m decreases the anchorage independent growth.

Previously, we found that WWP1 knockdown by siRNA inhibits cell proliferation in PC-3 (4), but not in LNCaP, C4-2, LAPC-4, and DU145 prostate cancer cell lines. However, WWP1 knockdown by siRNA induces apoptosis in two breast cancer cell lines (MCF7 and HCC1500) (1). We use the pSIH1-H1-Puro lentiviral system and obtained WWP1 knockdown MCF7 cell populations. We found that knockdown of WWP1 can sensitize these cells to TRAIL (**Fig. 4**). Similar results were observed in PC-3 (data not shown).

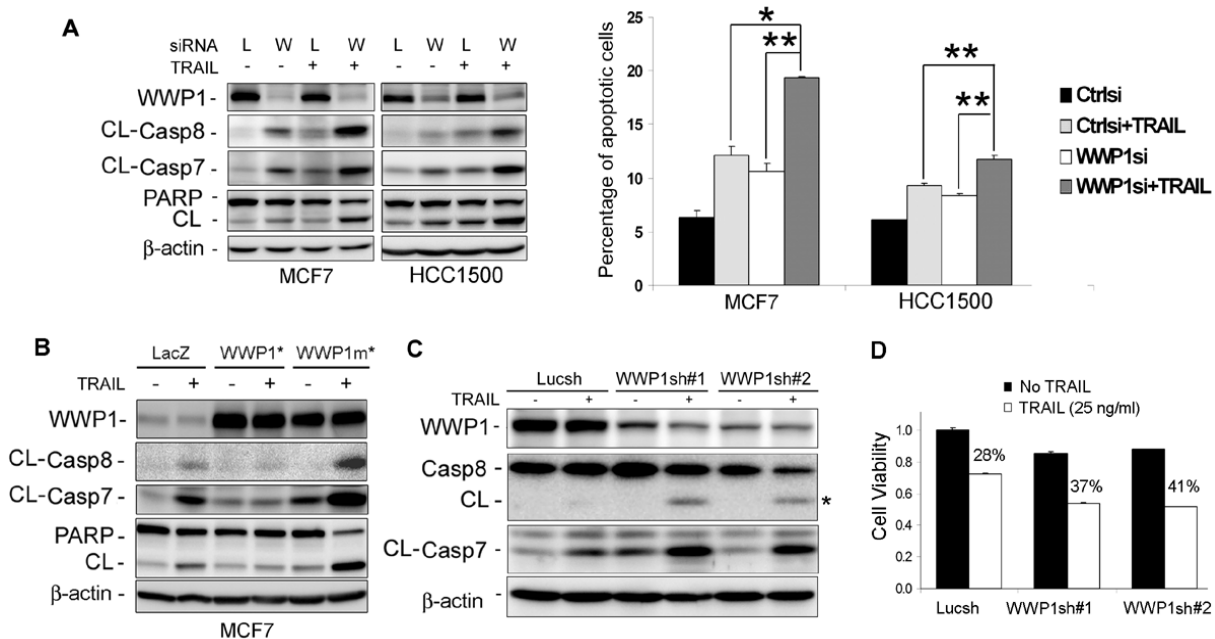


Fig. 4. The inhibition of WWP1 increased the TRAIL sensitivity in TRAIL-resistant cancer cell lines. **A.** WWP1 KD by siRNA and TRAIL (25 ng/ml) additively induced apoptosis in MCF7 and HCC1500 as detected by WB and flow cytometry. The cells were transfected with siRNAs for two days and were treated with TRAIL for one day. Apoptosis is indicated by the cleavage of caspase-8, -7 and PARP in the left panel. The right panel shows the quantitative apoptotic data (sub-G1 cells), as detected by the PI staining and flow cytometry, *, $P < 0.05$; **, $P < 0.01$ (t-test). **B.** The overexpression of WT WWP1* reduced, but WWP1m* increased, the TRAIL (25 ng/ml) -induced apoptosis in MCF7. Apoptosis is indicated by the cleavage of caspase-8, -7 and PARP. **C.** WWP1 KD by two different shRNA and TRAIL (25 ng/ml) additively induced apoptosis in MCF7 as detected by WB. Apoptosis is indicated by the cleavage of caspase-8 and -7. The 41/43 KDa cleaved caspase-8 bands are shown (*). **D.** WWP1 KD by two different shRNAs and TRAIL additively decreased the cell viability in MCF7 as detected by the SRB assay. The cells were treated with 25 ng/ml TRAIL for two days. The killing effects of TRAIL are shown.

In summary, we successfully established WWP1 overexpression 22Rv1 and MCF7 stable clones and WWP1 depletion MCF7 and PC-3 stable populations and studied their growth rate, apoptosis, and TRAIL response.

We first successfully expressed WWP1 using lentivirus in PZ-HPV7, RWPE1, 22Rv1, MCF10A, MCF7, and MDA-MB-231 cell lines. We found that WWP1 overexpression promoted cell proliferation in an E3 ligase activity independent manner (**Oncogene, 2008**). WWP1 overexpression in MCF7 protected cells from TRAIL-induced apoptosis; however, the E3 ligase dead mutant WWP1 overexpression promoted TRAIL-induced apoptosis (**Int. J. Cancer, 2011**).

In addition, we demonstrated that WWP1 is induced by TGF-beta in PC-3 and HaCaT cells. WWP1 is degraded through proteasome in 22Rv1. (**Cancer Metastasis Rev, 2007**). We collaborated with Lianping Xin at Rochester University and demonstrated that WWP1 suppresses SDF-1-induced cell migration and invasion (submitted).

We already completed this specific aim and published three papers.

- 1) [Ceshi Chen*](#), Yi Li, Zhongmei Zhou, Arun K. Seth, The WW domain containing E3 ubiquitin protein ligase 1 upregulates ErbB2 and EGFR through RING finger protein 11, **Oncogene**, 2008, 27: 6845-55.
- 2) Zhongmei Zhou, Rong Liu, [Ceshi Chen*](#), The WWP1 ubiquitin E3 ligase increases TRAIL resistance in breast cancer, **Int J Cancer**. 2011 Apr 7. [Epub ahead of print]
- 3) [Chen C*](#) and Matesic LE. 2007. The Nedd4-like family of E3 ubiquitin ligases and cancer (review). **Cancer Metastasis Rev** 26:587-604

Task 2. To determine the molecular mechanism of WWP1 promoting human prostate carcinogenesis:

1. Examine protein interaction among WWP1, RNF11, and Smad4 by Co-IP, GST-pull down assays, and immuno-fluorescence staining;
2. Check Smad4 ubiquitination and degradation by WWP1 through expressing RNF11 cDNA or siRNA;
3. Investigate expression of RNF11 in the prostate cancer samples by real-time PCR, northern blot, and western blots;
4. Co-transfect RNF11 and WWP1 cDNA or siRNA in prostate cancer and examine the TGF-beta response and cell growth;
5. Examine the protein expression of the WWP1 targets in cell models established in Aim 1 by western blot;
6. Perform rescue experiments through introducing mutant or siRNA for verified WWP1 substrates into cell models established in Aim 1

We firstly demonstrated that WWP1 forms a protein complex with RNF11, a negative regulator of ErbB2 and EGFR. The protein-protein interaction is through the first and third WW domains of WWP1 and the PY motif of RNF11 (**Fig. 5**).

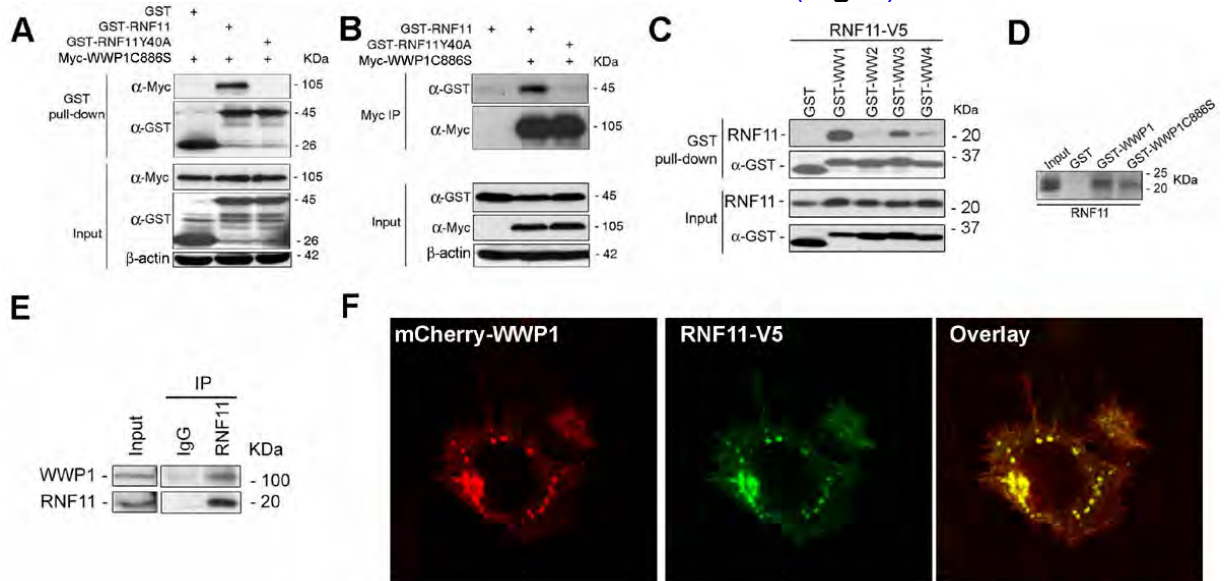


Fig. 5. WWP1 interacts with RNF11 via the WW/PY motifs. **A.** The PY motif of RNF11 is required for protein interaction with WWP1, as determined by the GST pull-down assay. Myc-WWP1C886S and GST-RNF11/GST-RNF11Y40A/GST were transfected into HEK293T for two days. GST fusion proteins were pulled down with Glutathione-Sepharose 4B slurry beads. **B.** The PY motif of RNF11 is required for protein interaction with WWP1, as determined by the co-IP experiment. Myc-WWP1C886S was immunoprecipitated with anti-Myc Ab and protein A beads. **C.** RNF11 binds to the first and third WW domains of WWP1.

The GST pull-down assay was performed. RNF11-V5 was probed with anti-V5 Ab. **D.** The recombinant GST-WWP1 and GST-WWP1C886S fusion proteins pulled down the in vitro translated ³⁵S-labeled RNF11 protein but the GST protein did not. **E.** Endogenous RNF11 interacts with WWP1 in MCF7. The MCF7 cell lysate from one 100-mm dish was immunoprecipitated with either 5 µl mouse anti-RNF11 antibody (Abnova) or mouse IgG. The blot was probed with rabbit anti-WWP1 and anti-RNF11 Abs. 5% of the input cell lysate was used as the control. **F.** Co-localization of WWP1 and RNF11 in HEK293T cells. RNF11-V5 was detected by immunofluorescence staining using anti-V5 Ab. Myc-mCherry-WWP1 can be directly visualized under the fluorescent microscope.

Although WWP1 is able to ubiquitinate RNF11 in vitro and in vivo, WWP1 neither targets RNF11 for degradation nor changes RNF11's cellular localization (**Fig. 6**).

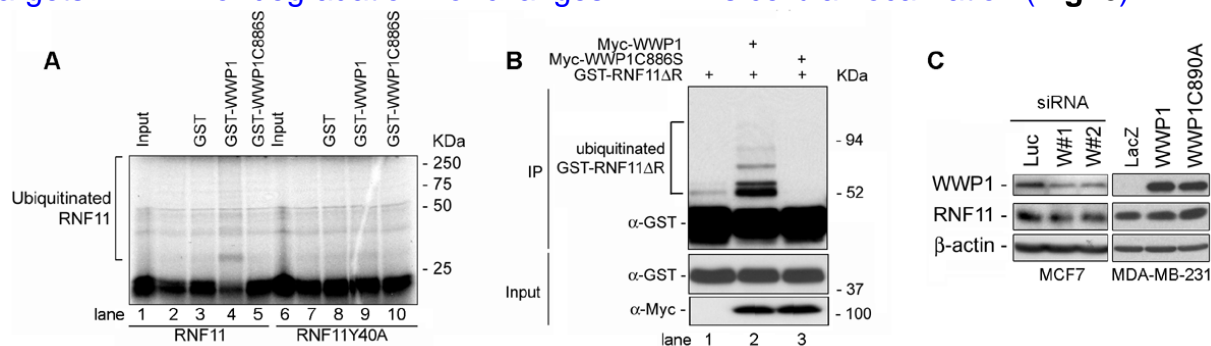


Fig. 6. WWP1 ubiquitinates RNF11 but does not regulate the RNF11 protein stability. A.

The recombinant GST protein or GST-fused WWP1/WWP1C886S proteins were incubated with in vitro translated RNF11 or RNF11Y40A proteins labeled with ³⁵S, and the reaction products were subjected to PAGE and autoradiography. "Input" is in vitro translated RNF11 (lane 1) or RNF11Y40A (lane 6) proteins which serve as negative controls. Lanes 2 and 7 also serve as negative controls because only the ubiquitin conjugation reagents but not any GST recombinant proteins were added into the reactions. **B.** WT but not catalytic inactive WWP1C886S ubiquitinates GST-RNF11ΔR in HEK293T cells. The GST-RNF11ΔR proteins were pulled down by Glutathione Sepharose 4B beads and detected by anti-GST Ab. **C.** WWP1 does not affect the stable levels of endogenous RNF11 in breast cancer cells. Two WWP1 siRNAs were transfected into MCF7.

Importantly, inhibition of RNF11 can rescue WWP1 siRNA-induced ErbB2 and EGFR downregulation and growth arrest. Finally, we demonstrated that RNF11 is overexpressed in a panel of prostate cancer cell lines with WWP1 expression (**Fig. 7**). These findings suggest that WWP1 may promote cell proliferation and survival partially through suppressing RNF11-mediated ErbB2 and EGFR downregulation.

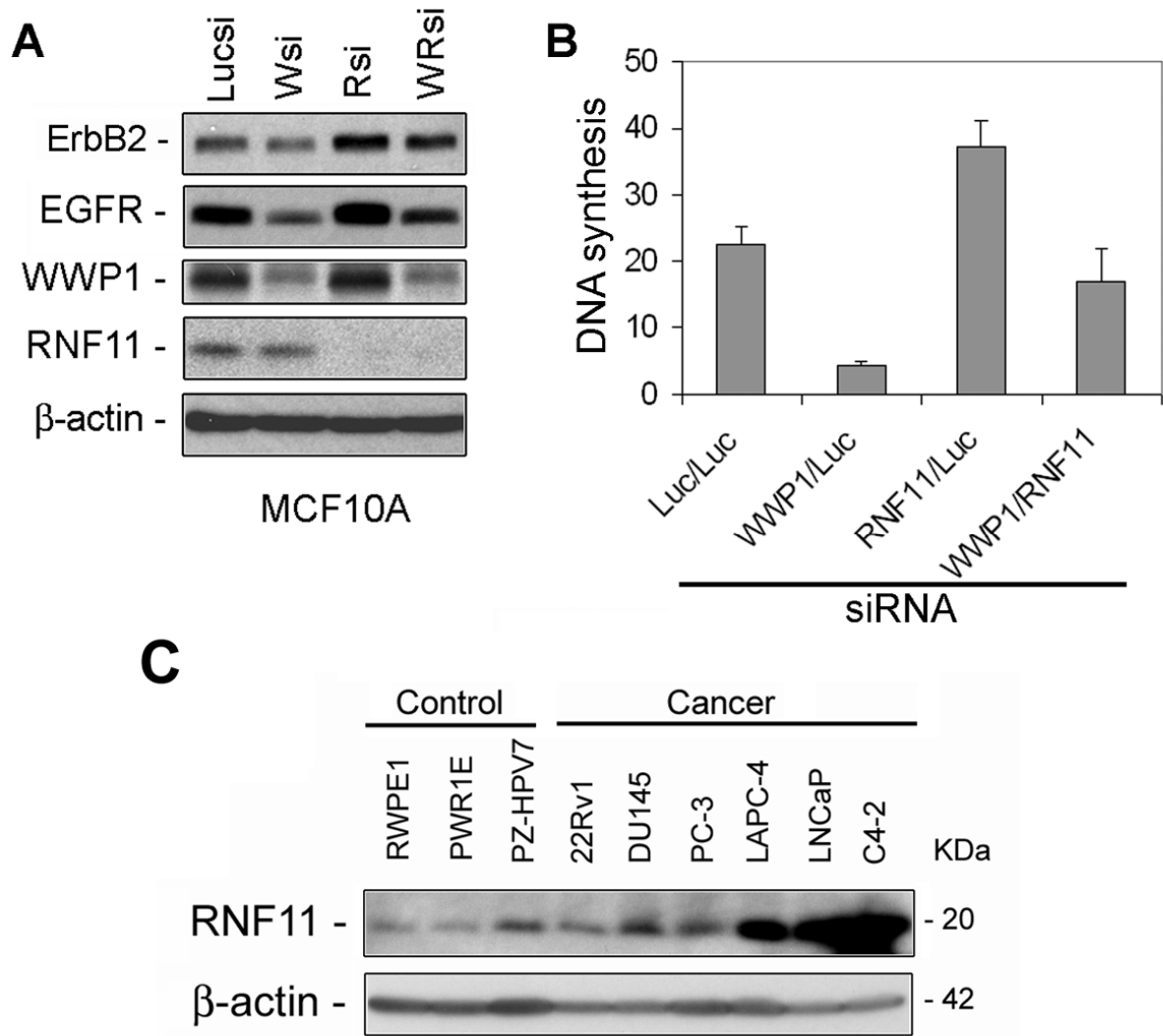


Fig. 7. RNF11si rescues the WWP1si-induced ErbB2 and EGFR downregulation and growth arrest in MCF10A. **A.** RNF11 siRNA#1 effectively rescues WWP1 siRNA#1 induced ErbB2 and EGFR decrease in MCF10A, as measured by Western blot. **B.** RNF11si#1 effectively rescues WWP1 siRNA#1 induced DNA synthesis decrease in MCF10A, as measured by 3H-thymidine incorporation. Both WWP1 siRNA and RNF11 siRNA were transfected at 100 nM final concentration for 48 hrs. **C.** The RNF11 protein level is up-regulated in prostate cancer cell lines compared to three immortalized prostate epithelial cell lines, as determined by Western blot.

Additionally, we demonstrated that WWP1 targets the p63 transcription factor and the ErbB4 receptor for ubiquitin-mediated proteasomal degradation and regulates apoptosis (data not shown). Furthermore, we continued to study the WWP1 mediated KLF5 protein degradation (6). We found that another E3 ubiquitin ligase Fbw7 can also target KLF5 for degradation (data not shown). Thus, WWP1 and Fbw7 coordinately target KLF5 for degradation.

In summary, WWP1 E3 ligases function through ubiquitinating RNF11, KLF5, p63, and ErbB4. We published additional four papers.

- 1) Yi Li, Zhongmei Zhou, [Ceshi Chen*](#), WW domain containing E3 ubiquitin protein ligase 1 targets p63 transcription factor for ubiquitin-mediated proteasomal

degradation and regulates apoptosis, **Cell death and differentiation**, 2008, 15: 1941-51

- 2) Yi Li, Zhongmei Zhou, Maurizio Alimandi, Ceshi Chen* WW domain containing E3 ubiquitin protein ligase 1 targets the full length ErbB4 for ubiquitin-mediated degradation in breast cancer, **Oncogene**, 2009 Aug 20;28(33):2948-58
- 3) Zhao D, Zheng H, Zhou Z, Ceshi Chen*. The Fbw7 tumor suppressor targets KLF5 for ubiquitin-mediated degradation and suppresses breast cell proliferation. **Cancer Res**, 2010, 70(11):4728-4738.
- 4) Rong Liu, Zhongmei Zhou, Dong Zhao, Ceshi Chen*, The Induction of KLF5 Transcription Factor by Progesterone Contributes to Progesterone-Induced Breast Cancer Cell Proliferation and Dedifferentiation, **Mol Endocrinol**, 2011 May 12. [Epub ahead of print] (Cover article)

Task 3. To test the hypothesis that WWP1 over-expression causes prostate cancer in vivo by establishing a WWP1 prostate transgenic mouse model:

1. Construct and evaluate the ARR₂PB-myc-WWP1 expression vector in LNCaP;
2. Generate and validate ARR₂PB-myc-WWP1 transgenic mice;

We failed to obtain prostate specific WWP1 Tg mice after several attempts. We already replace this aim with the following aim with the approval of DoD PCRP.

Task 3. To test whether PMEPA1 (a WWP1 interacting protein) can promote androgen receptor negative prostate cancer cell cycle through inhibiting CDK inhibitor p21 expression:

The PMEPA1 gene has been shown to suppress the androgen receptor (AR) and TGF β signaling pathways and is abnormally expressed in prostate tumors. However, the role and mechanism action of PMEPA1 in AR-negative prostate cancer are unclear. we firstly demonstrate that inhibition of PMEPA1 suppresses AR-negative RWPE1 and PC-3 prostate cell proliferation through upregulating the p21 transcription (**Fig. 8**).

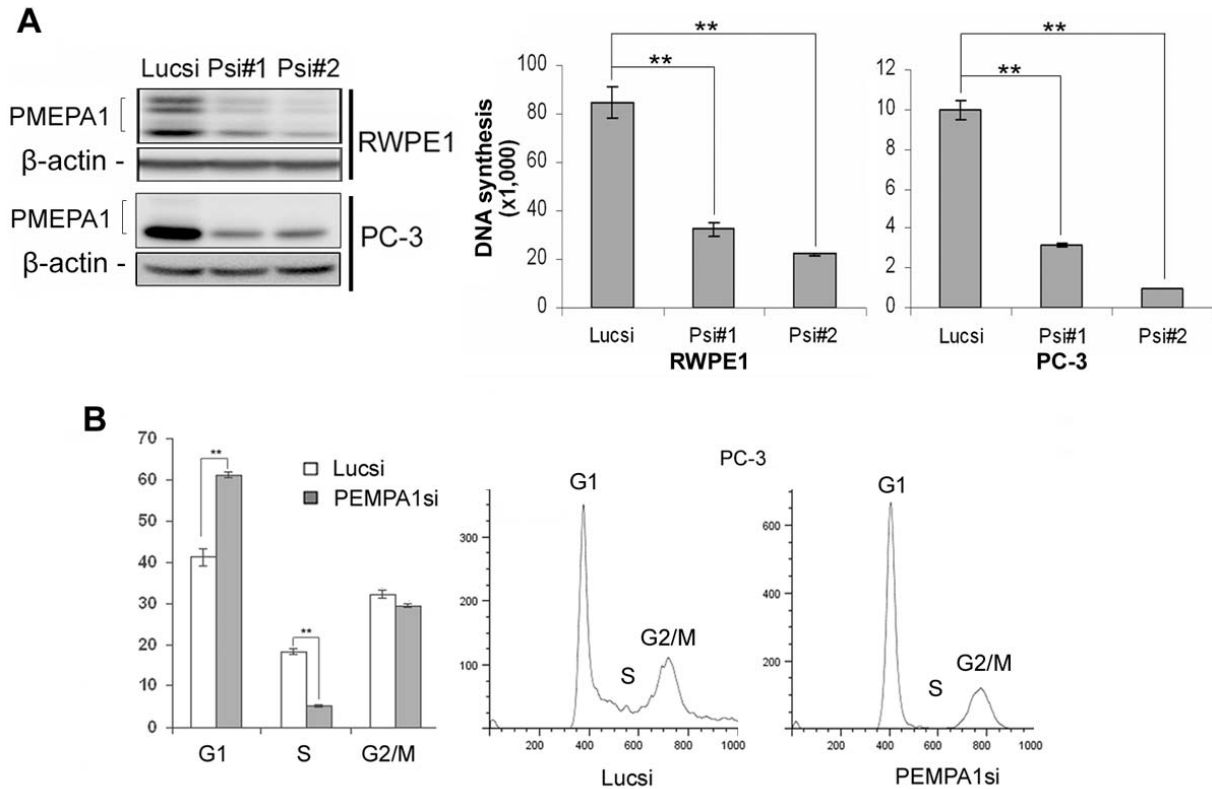


Fig. 8. Depletion of PMEPA1 inhibits AR-negative prostate cell proliferation through blocking the G1/S cell cycle transition. **A.** Depletion of PMEPA1 inhibits 3H-thymidine incorporation in both RWPE1 and PC-3 prostate cell lines. Knockdown of the PMEPA1 protein expression by two different siRNAs in RWPE1 and PC-3 was examined by immunoblotting (left panel). **B.** Knockdown of PMEPA1 by siRNA#2 in PC-3 prevents the G1/S cell cycle progression by the PI staining and flow cytometry analysis. The average results from the three experiments are shown on the left side. **, $p \leq 0.01$ (t-test). One example result of flow cytometry is shown on the right side.

Additionally, PMEPA1 overexpression suppresses the p21 expression and promotes cell proliferation (data not shown). PMEPA1 is induced by TGF β as a negative feedback loop to suppress Smad3 phosphorylation and nuclear translocation, upregulates c-Myc, downregulates p21, and promotes PC-3 cell proliferation. The PMEPA1 functions depend on its Smad2/3 binding motif. Consistently, depletion of Smad3/4, but not Smad2, blocks the PMEPA1's functions of regulating c-Myc and p21 (**Fig. 9**).

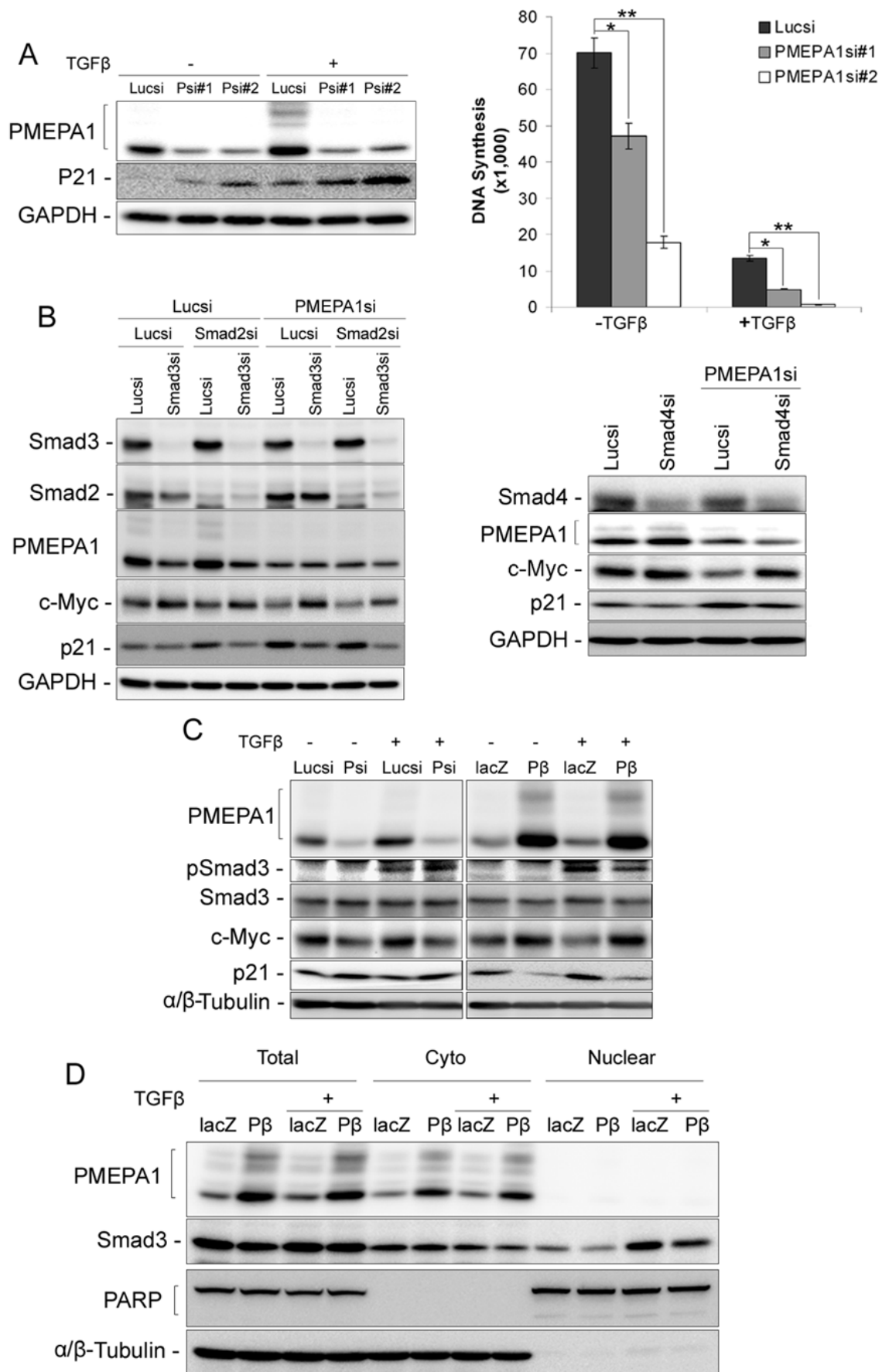


Fig. 9. PMEPA1 suppresses the p21 expression and cell proliferation through blocking Smad3 nuclear translocation in the presence and absence of TGF β . **A.** Knockdown of PMEPA1 increases the TGF β induced p21 expression in PC-3, as determined by immunoblotting (left panel). PC-3 cells were transfected with different PMEPA1 siRNAs. On the second day after transfection, the cells were serum starved overnight and treated with 2 ng/ml TGF β for 6 hours. Knockdown of PMEPA1 increases the TGF β induced PC-3 growth arrest, as determined by the DNA synthesis assay (right panel). *, P<0.05; **, P<0.01, t-test. **B.** Knockdown of Smad3 but not Smad2 rescues PMEPA1 siRNA#1 induced c-Myc downregulation and p21 upregulation in PC-3 (left panel). Knockdown of Smad4 rescues PMEPA1 siRNA#1 induced c-Myc downregulation and p21 upregulation in PC-3 (right panel). **C.** Knockdown of PMEPA1 increases and overexpression of PMEPA1 β decreases the Smad3 phosphorylation in the presence of TGF β (2 ng/ml, 1 hour) in PC-3. **D.** Overexpression of PMEPA1 β in PC-3 decreases the nuclear Smad3 protein levels in the absence and presence of TGF β (2 ng/ml, 1 hour). PARP is a nuclear marker; and α/β -Tubulin is the cytoplasm marker.

Importantly, stable depletion of PMEPA1 in PC-3 inhibits xenograft growth (**Fig. 10**).

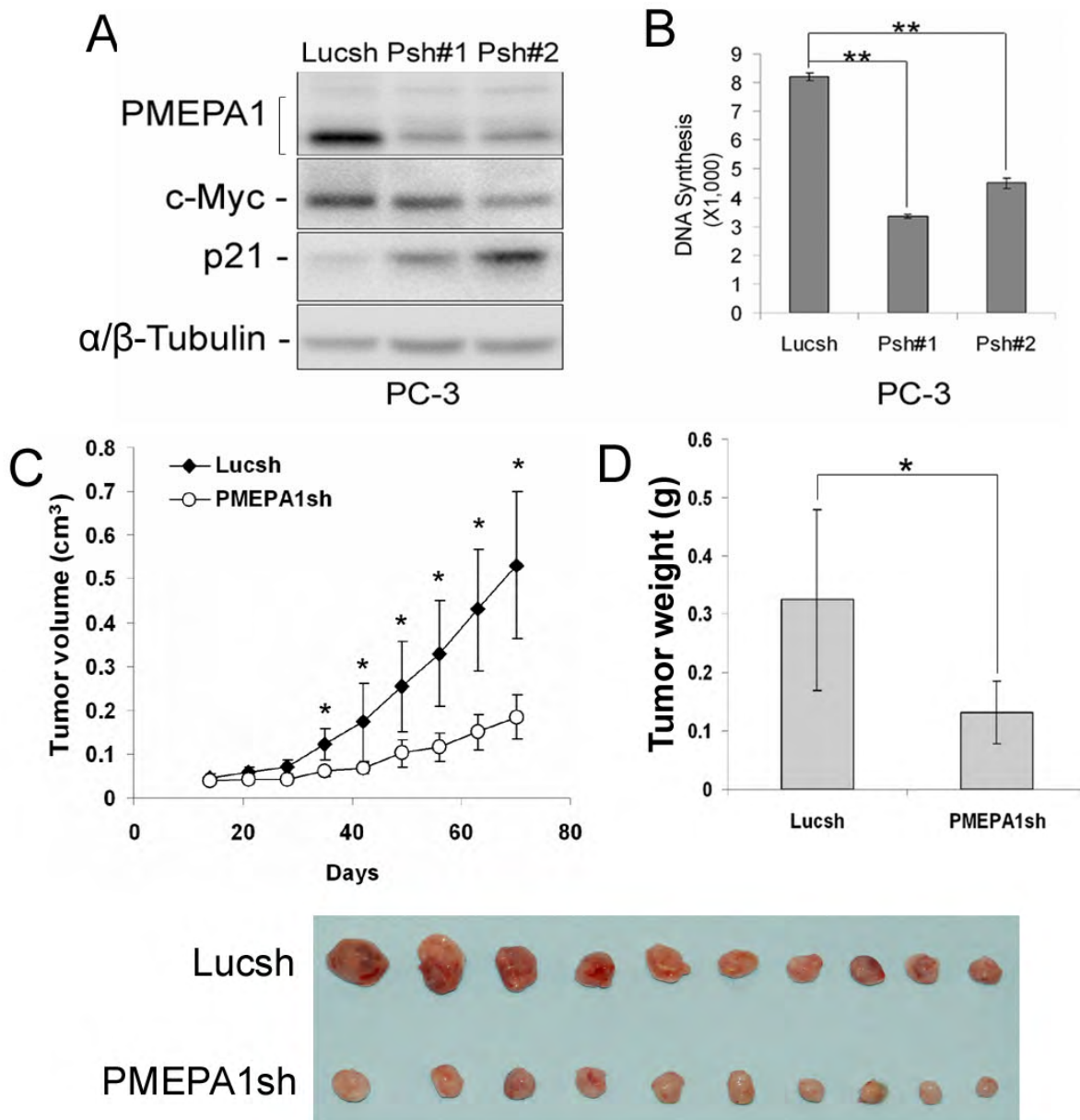


Fig. 10. Depletion of PMEPA1 inhibits PC-3 prostate cancer growth in vivo

A. Stable knockdown of PMEPA1 downregulates the c-Myc protein expression and upregulates the p21 protein expression in PC-3. **B.** Stable knockdown of PMEPA1 in PC-3 significantly suppresses DNA synthesis in vitro. **C.** The PC-3 PMEPA1sh#2 cell population grows more slowly than the Lucsh control cell population in male SHO mice. *, $p < 0.05$ (t-test). **D.** Stable knockdown of PMEPA1 significantly decreases the xenograft tumor weight ($n=10$). *, $p < 0.05$ (t-test). The tumors were harvested at day 70.

Finally, we found that PMEPA1 is overexpressed in a subset of prostate cancer cell lines and tumors (**Fig. 11**).

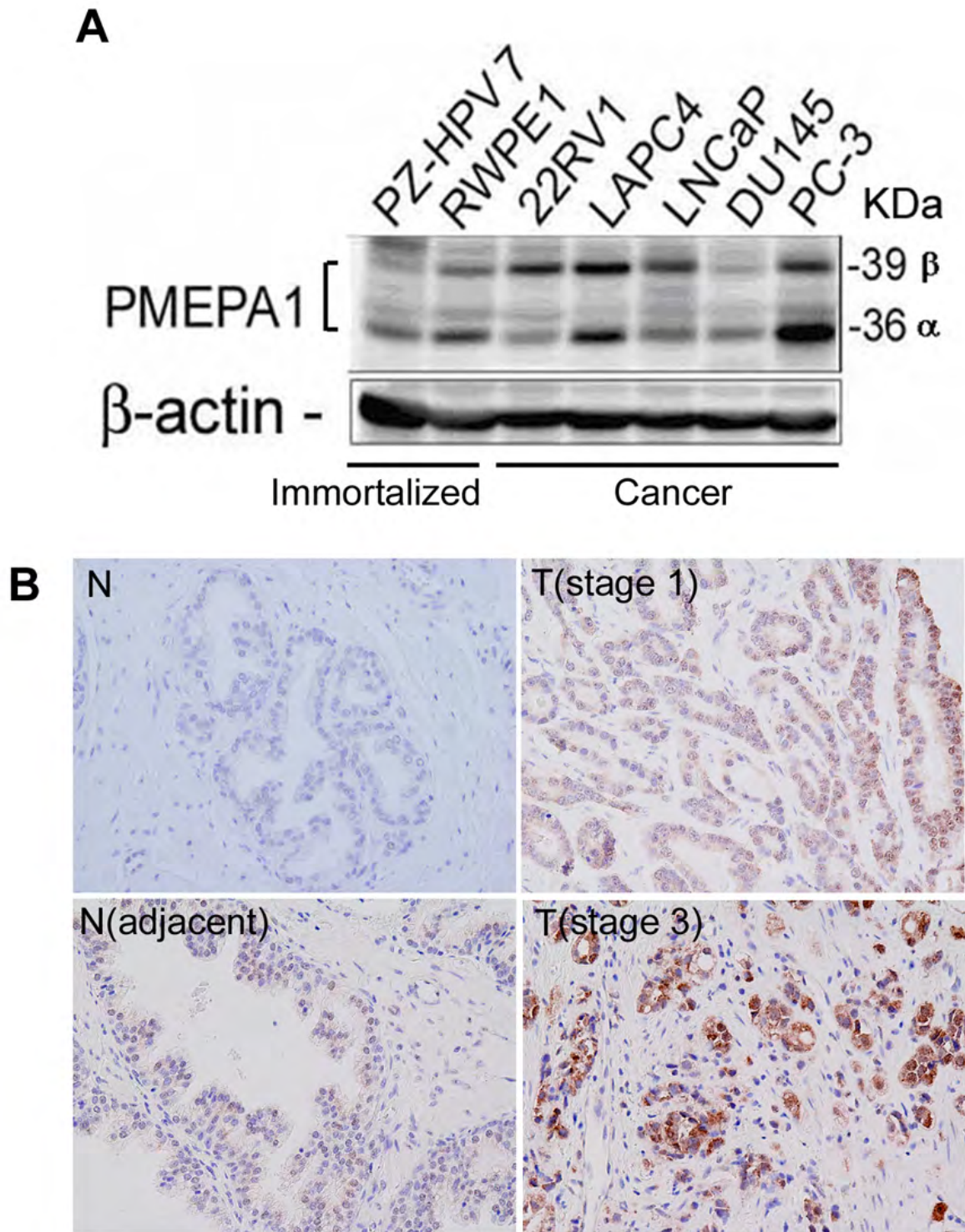


Fig. 11. PMEPA1 is overexpressed in prostate cancer and the pathway by which PMEPA1 inhibits cell proliferation. **A.** The protein expression of PMEPA1, both 36 kDa α and 39 kDa β isoforms, in the immortalized prostate epithelial cell lines (PZ-HPV7 and RWPE1) and five prostate cancer cell lines was measured by immunoblotting. β -actin served as the loading control. **B.** Examples of IHC staining for PMEPA1 in normal prostate (N: score 0), adjacent normal (score 3), and prostate carcinomas (T, stage 1: score 6, and stage 3: score 8).

These findings suggest that PMEPA1 may promote AR-negative prostate cancer cell

proliferation through p21. We already completed this specific aim and published one paper.

Rong Liu, Zhongmei Zhou, Jian Huang, [Ceshi Chen](#)*, PEMPA1 promotes androgen receptor negative prostate cell proliferation through suppressing the Smad3/4-c-Myc-p21Cip1 signaling pathway, **J. Pathology**, 2011, 223(5):683-94

Key Research Accomplishments

1. WWP1 depletion suppresses cancer cell proliferation and promotes apoptosis
2. WWP1 suppresses cell migration and invasion
3. WWP1 interacts with RNF11 and inhibits RNF11-mediated EGFR and ErbB2 downregulation
4. WWP1 targets p63 transcription factor for ubiquitin-mediated proteasomal degradation and regulates cell apoptosis
5. WWP1 targets ErbB4 for ubiquitin-mediated degradation
6. WWP1 increases the TRAIL resistance through Caspase 8
7. WWP1 and Fbw7 coordinately target KLF5 for ubiquitin-mediated degradation.
8. WWP1 mediated KLF5 ubiquitination and degradation is inhibited by the YAP and TAZ protein.
9. The WWP1 interacting protein PMEPA1 promotes prostate cell cycle progression and regulating Myc and p21.
10. WWP1 is overexpressed in ER and IGF-1R positive breast tumors by IHC study.
11. KLF5 promotes cell survival through FGF-BP-ERK-MKP-1
12. KLF5 contributes to progesterone-induced cell proliferation and de-differentiation
13. The HECTD3 E3 ubiquitin ligase promotes prostate cancer cell survival

WWP1 appears to be a context dependent oncogene in cancers. The WWP1 mRNA and protein levels are frequently up-regulated in a subset of prostate and breast cancers (1, 4). Interestingly, the expression of WWP1 in breast tumors correlates with positive ER α and insulin-like growth factor 1 receptor (IGF-1R) statuses, which are good prognosis biomarkers in breast tumors. In MCF10A breast cells, when WWP1 was knocked down by siRNA, the cells became more resistant to doxorubicin-induced apoptosis (7). This result suggests that WWP1 has a pro-apoptotic function in MCF10A. The WWP1 pro-apoptotic function can be attributed to targeting several pro-survival proteins, such as Δ Np63 (7) and KLF5 (6), for ubiquitin-mediated degradation. Consistently, the expression of WWP1 is negatively correlated with the expression of Δ Np63 (7) and KLF5 (8) in breast cancers.

In contrast to the WWP1 pro-apoptotic function in ER α -negative breast cells, WWP1 depletion in ER α -positive MCF7 and HCC1500 breast cancer cell lines suppressed cell proliferation and induced apoptosis (1, 9-11). Similar results were obtained in MDCK (2), PC-3 (4), and HCT116 cells (7). In addition, WWP1 overexpression promoted breast epithelial cell growth and anchorage-independent growth (11). WWP1 enhances cell proliferation and survival likely through both ubiquitin ligase-dependent and -independent activities. In MDCK cells, WWP1 suppressed the expression of T β R1, Smad2, and TGF β -induced PAI1 and JunB (2). In PC-3, WWP1 suppressed the expression of T β R1, Smad4, and the cell cycle dependent kinase inhibitor p15 (4). In HCT116, WWP1 targeted the TAp63 for degradation (7). In addition, WWP1 enhances MAPK signaling through decreasing the ErbB2 and EGFR turnover (9). Thus, WWP1 may have a context dependent role in cancer development.

To conclusively sort this out, the physiological role of WWP1 needs to be elucidated in animal models.

Reportable Outcomes

1. Twelve papers (PI is the corresponding author for all these papers)

- 1) [Chen, C*](#) and Matesic, L. E. The Nedd4-like family of E3 ubiquitin ligases and cancer. **Cancer Metastasis Rev**, 2007,26: 587-604.
- 2) [Ceshi Chen*](#), Yi Li, Zhongmei Zhou, Arun K. Seth, The WW domain containing E3 ubiquitin protein ligase 1 upregulates ErbB2 and EGFR through RING finger protein 11, **Oncogene**, 2008, 27: 6845-55.
- 3) Yi Li, Zhongmei Zhou, [Ceshi Chen*](#), WW domain containing E3 ubiquitin protein ligase 1 targets p63 transcription factor for ubiquitin-mediated proteasomal degradation and regulates apoptosis, **Cell death and differentiation**, 2008, 15: 1941-51
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- 8) Jin-Tang Dong*, [Ceshi Chen*](#), Essential role of KLF5 transcription factor in cell proliferation and differentiation and its implications for human diseases, (Review), **Cellular and Molecular Life Sciences**, 2009 Aug;66(16):2691-706.
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- 10) Rong Liu, Zhongmei Zhou, Dong Zhao, [Ceshi Chen*](#), The Induction of KLF5 Transcription Factor by Progesterone Contributes to Progesterone-Induced Breast Cancer Cell Proliferation and Dedifferentiation, **Mol Endocrinol**, 2011 May 12. [Epub ahead of print] (Cover article)
- 11) Zhongmei Zhou, Rong Liu, [Ceshi Chen*](#), The WWP1 ubiquitin E3 ligase increases TRAIL resistance in breast cancer, **Int J Cancer**. 2011 Apr 7. [Epub ahead of print]
- 12) Xu Zhi, [Ceshi Chen*](#), WWP1: a versatile ubiquitin E3 ligase in signaling and diseases (review), **submitted**, 2011.

2. Twenty-one abstracts

- 1) Li Y, Zhou Z, [Chen C](#). WWP1 regulates cell apoptosis through targeting p63 for ubiquitin-mediated proteasomal degradation, International Symposium on Protein Modification and Degradation in Beijing (SPMDB). Nov. 4-7, 2007
- 2) [Chen C](#), Li Y, Zhou Z, Seth AK. WWP1 promotes cell proliferation through blocking RNF11 mediated Erbb2 and EGFR downregulation [abstract]. In: Proceedings of the 99th Annual Meeting of the American Association for Cancer

Research; 2008 Apr 12-16; San Diego, CA. Philadelphia (PA): AACR; 2008. Abstract nr 140

- 3) Li Y, Zhou Z, Chen C. HECTD3: A new e3 ligase upregulated in breast and prostate cancer [abstract]. In: Proceedings of the 99th Annual Meeting of the American Association for Cancer Research; 2008 Apr 12-16; San Diego, CA. Philadelphia (PA): AACR; 2008. Abstract nr 1790
- 4) Hanqiu Zheng, Zhongmei Zhou, and Ceshi Chen, A 9-amino acid Motif at KLF5 Nterminus Contributes to WWP1 E3 Ubiquitin Ligase Mediated KLF5 Protein Degradation. The first international symposium on the biology of the kruppel-like factors, University of Tokyo auditorium (Tokyo, Japan), March 6-7 of 2008
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- 14) Han-Qiu Zheng, Dong Zhao, Zhongmei Zhou, Ceshi Chen, The SCF^{Fbw7} E3 ubiquitin ligase complex targets KLF5 for ubiquitination and degradation in a phosphorylation dependent manner, Proceedings of the American Association for Cancer Research; 2010 Apr; Abstract nr 3150
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3. **Twenty-one oral presentations**

- 1) Identification of E3 ubiquitin ligases for cancer target therapy, Cancer Institute, Chinese Academy of Medical Sciences, East China Normal University, Central South University, and Nankai University, Nov, 2007
- 2) WWP1 regulates cell apoptosis through targeting p63 for ubiquitin-mediated proteasomal degradation, International Symposium on Protein Modification and Degradation in Beijing (SPMDB). Nov. 4-7, 2007
- 3) E3 ubiquitin ligases for cancer target therapy, Shanghai Jiaotong University, Hunan normal university, Mar 2008
- 4) E3 ubiquitin ligases with genetic alterations for cancer target therapy, Central South University, China, Oct. 2008
- 5) E3 ubiquitin ligases with genetic alterations for cancer target therapy, Nankai University, China, Oct. 2008
- 6) The ubiquitination and proteasomal degradation of KLF5, The first international symposium on the biology of the kruppel-like factors, University of Tokyo auditorium (Tokyo, Japan), Mar 7, 2008
- 7) E3 ubiquitin ligases with genetic alterations for prostate cancer target therapy, Daping Hospital, Third Military Medical University, Chongqing, China, Oct. 2008
- 8) E3 ubiquitin ligases with genetic alterations for cancer target therapy, Tianjing General Hospital, China, Oct. 2008
- 9) E3 ubiquitin ligases with genetic alterations for cancer target therapy, GE Research Center, Niskayuna, New York, USA, Oct 15, 2008

- 10) The KLF5 transcription factor in cancer, Rensselaer Polytechnic Institute, Albany, Jun 2008
- 11) Identification of E3 ubiquitin ligases for cancer target therapy, Cancer Institute, Southern Illinois University, Springfield, IL, Jan 23, 2009
- 12) The role of KLF5 transcription factor in breast cancer, Capital Region Cancer Research Meeting, Albany, NY, Feb 12, 2009
- 13) The E3 ubiquitin ligases as Novel Molecular Targets in Breast Cancer, BIT's 2nd Annual World Cancer Congress; Beijing, China, 2009 Jun 22-25
- 14) The role of KLF5 transcription factor in cancer, Gen*NY*Sis Center for Excellence in Cancer Genomics, University at Albany, Albany, NY, Aug 21, 2009
- 15) The role and regulation of KLF5 transcription factor in cancer, Beijing Cancer Hospital, Peking University, Beijing, China, Nov 24, 2009
- 16) Targeting the KLF5 Transcription Factor for Ubiquitin Proteasome Degradation in Cancer, kaohsiung medical university, TW, Apr 30, 2010
- 17) The role and regulation of KLF5 in cancer, Institute of Biomedical Sciences, East China Normal University, Apr 28, 2010
- 18) Identification of E3 ubiquitin ligases for cancer diagnosis and therapy, Ordway Research Institute, Albany, Jun 24, 2010
- 19) E3 ubiquitin ligases for cancer targeted therapy, Sunnybrook Health Science Center, Toronto, CA, Jul 26, 2010
- 20) Targeting the KLF5 Transcription Factor for Ubiquitin Proteasome Degradation in Breast Cancer, BIT's 3rd Annual World Cancer Congress Breast Cancer Conference; Shanghai, China, Apr 25-27, 2010
- 21) The emerging role of KLF5 transcription factor in Hippo tumor suppressor pathway, Nankai University, China, Apr 28, 2011

4. Cell lines and mouse models

- 1) WWP1 stable knockdown PC-3 and MCF7 cell lines
- 2) WWP1 overexpressing 22Rv1 cell lines
- 3) Klf5 conditional knockout mouse model
- 4) HECTD3 knockout mouse model

5. Funding applied for based on work supported by this award

- 1) American Cancer Society (ACS), Molecular Genetics and Oncogenes (MGO) "The Role of the WWP1 E3 Ubiquitin Ligase in Human Breast Cancer". (direct cost), July 2008 - Jun, 2012.
- 2) Department of Defense (DOD), BCRP, BC075738, WWP1 targets ErbB4 for ubiquitin-mediated degradation in breast cancer. (direct cost), Sep 2008- Aug 2009.
- 3) The American Urological Association Foundation (AUA) postdoctoral fellowship (Mentor for Dr. Liu, Rong). (direct cost), Jul 2009- Jul 2011

6. Employment or research opportunities

Five postdoctoral fellows have been hired for this project. Additionally, one Ph.D. graduate student, two MD students, and three undergraduate students have accepted training in the lab during this period.

Conclusion

In conclusion, the completed research helps us better understand the role of WWP1 and mechanism of WWP1 action in prostate cancer. In addition, we discovered that PMEPA1 is an attractive therapeutic target in AR negative prostate cancer and HECTD3 is a new oncogenic E3 ligase in human prostate cancer. The knowledge will help us design better strategy for prostate cancer targeted therapy.

With the support of this award and others, we delivered 12 manuscripts, 21 meeting abstracts, and 21 oral presentations in last four years. Based on this award, we were awarded an ACS grant and a DoD BCRP concept grant. One postdoctoral fellow was awarded the AUA fellowship. One Ph.D. graduate student was graduated.

The PI deeply appreciated the funding from DoD PCRCP and acknowledged the funding in all publications, abstracts, and oral presentations. The PI thinks that this award is fruitful and looks forward to more collaboration in the future.

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Appendices

Papers published

- 1) [Chen, C*](#) and Matesic, L. E. The Nedd4-like family of E3 ubiquitin ligases and cancer. **Cancer Metastasis Rev**, 2007,26: 587-604.
- 2) [Ceshi Chen*](#), Yi Li, Zhongmei Zhou, Arun K. Seth, The WW domain containing E3 ubiquitin protein ligase 1 upregulates ErbB2 and EGFR through RING finger protein 11, **Oncogene**, 2008, 27: 6845-55.
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The Nedd4-like family of E3 ubiquitin ligases and cancer

Ceshi Chen · Lydia E. Matesic

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Abstract Accumulating evidence suggests that E3 ubiquitin ligases play important roles in cancer development. In this article, we provide a comprehensive summary of the roles of the Nedd4-like family of E3 ubiquitin ligases in human cancer. There are nine members of the Nedd4-like E3 family, all of which share a similar structure, including a C2 domain at the N-terminus, two to four WW domains in the middle of the protein, and a homologous to E6-AP COOH terminus domain at the C-terminus. The assertion that Nedd4-like E3s play a role in cancer is supported by the overexpression of Smurf2 in esophageal squamous cell carcinoma, WWP1 in prostate and breast cancer, Nedd4 in prostate and bladder cancer, and Smurf1 in pancreatic cancer. Because Nedd4-like E3s regulate ubiquitin-mediated trafficking, lysosomal or proteasomal degradation, and nuclear translocation of multiple proteins, they modulate important signaling pathways involved in tumorigenesis like TGF β , EGF, IGF, VEGF, SDF-1, and TNF α . Additionally, several Nedd4-like E3s directly regulate various cancer-related transcription factors from the Smad, p53, KLF, RUNX, and Jun families. Interestingly, multiple Nedd4-like E3s show ligase independent function. Furthermore, Nedd4-like E3s themselves are frequently regulated by phosphorylation, ubiquitination, translocation, and transcription in cancer cells. Because the regulation and biological output of these E3s is such a complex process, study of the role of these E3s in cancer

development poses some challenges. However, understanding the oncogenic potential of these E3s may facilitate the identification and development of biomarkers and drug targets in human cancer.

Keywords Nedd4 · WWP1 · Smurf · AIP4/Itch · E3 · Ubiquitination · Cancer

Abbreviations

AIP	atrophin-1 interacting protein
AR	androgen receptor
BMP	bone morphogenetic protein
CHX	cycloheximide
CIN85	Cbl-interacting protein of 85 KDa
CISK1	cytokine independent survival kinase 1
CXCR4	chemokine (C-X-C motif) receptor 4
DAT	dopamine transporter
E6-AP	E6 associated protein
HECT	homologous to E6-AP COOH terminus
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ENaC	epithelial sodium channel
EPS15	epithelial growth factor receptor substrate 15
FLIP	FLICE (pro-caspase-8)-inhibitory protein
GR	glucocorticoid receptor
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
IGF-1R	insulin-like growth factor 1 receptor
KLF	krüppel-like factor
MEF	mouse embryonic fibroblasts
mUb	mono-ubiquitination
MVB	multi-vesicular bodies
Nedd4	the neural precursor cells-expressed developmentally down-regulated 4
NGF	nerve growth factor

C. Chen (✉)
The Center for Cell Biology and Cancer Research,
Albany Medical College,
47, New Scotland Ave.,
Albany, NY 12208, USA
e-mail: chenc@mail.amc.edu

L. E. Matesic
Department of Biological Sciences, University of South Carolina,
Columbia, SC 29208, USA

PR	progesterone receptor
RING	the really interesting new gene
SCF	Skp1-Cul1-F-box; SDF-1: stromal cell-derived factor-1
SGK	serum and glucocorticoid-inducible kinase
Smurf	Smad ubiquitin regulatory factor
SOD1	superoxide dismutases-1
TβR1	TGF-β receptor 1
Tiul1	TGIF interacting ubiquitin ligase 1
TNFα	tumor necrosis factor-alpha
TRAP-δ	translocon-associated protein δ
TSG	tumor suppressor genes
UIM	ubiquitin interaction motif
UPS	ubiquitin proteasome system

1 Introduction

1.1 Ubiquitination, E3 ligases, and cancer

Protein ubiquitination is a post-translational modification that can direct proteins for degradation by the 26S proteasome or plasma membrane proteins for endocytosis, sorting, and destruction in the lysosome. Ubiquitination can also mediate proteolysis-independent effects like modulation of signal transduction, transcription, or DNA repair. The differing fates of ubiquitinated proteins depend on the length and architecture of ubiquitin chain [1]. Ubiquitin is an evolutionarily conserved 76 amino acid protein that is covalently conjugated to target proteins. K48-linked poly-ubiquitin chains are well known to function in protein degradation by the 26S proteasome. K63-linked poly-ubiquitin chains have been proposed to function in DNA repair, IκB kinase activation, translational regulation, and endocytosis [2]. Mono-ubiquitination (mUb) has been implicated in the endocytosis and trafficking of plasma membrane proteins [3]. Protein ubiquitination is generally catalyzed by the sequential activity of three enzymes: a ubiquitin activation enzyme (E1), ubiquitin conjugation enzymes (E2s), and ubiquitin ligases (E3s). It has been well established that E3s control the substrate specificity in this process. E3 ubiquitin ligases are important in cellular regulation because E3s specifically recognize a substrate for modification temporally and spatially. In mammalian cells, there are more than 500 E3s. Most single peptide E3s contain either a RING (the really interesting new gene) finger domain or a HECT (homologous to E6-AP COOH terminus) domain.

E3 ligases could be attractive targets for cancer therapy because of their substrate specificity. FDA approval of the general proteasome inhibitor, Velcade, for the treatment of multiple myeloma speaks to the promise of targeting the ubiquitin-proteasome system in anti-cancer therapy. How-

ever, proteasome inhibitors are not specific to cancer cells, and thus have obvious side effects. The E3 ligases could be better targets for cancer therapy due to their specificity [4]. Indeed, frequent genetic alterations and aberrations in the expression of E3s have been documented in human breast cancer [5]. Many E3s have been identified as either tumor suppressors (i.e., BRCA1 and Fbw7) or oncoproteins (i.e., Mdm2 and Skp2).

1.2 Structures and members of the Nedd4-like family

The *neural precursor cells-expressed developmentally down-regulated 4 (Nedd4)* gene encodes a HECT type E3 ligase with three functional domains: an N-terminal C2 domain for membrane binding, a central region containing WW domains for protein-protein interaction, and a C-terminal HECT domain for ubiquitin protein ligation [6] (Fig. 1). The C2 domain is a calcium-binding domain that is approximately 120 amino acids in length. Upon Ca^{2+} binding, the C2 domain can bind to phospholipids, inositol polyphosphates, and some proteins [7, 8]. WW domains are 35–40 amino acid long domains containing two conserved tryptophan (W) residues spaced 21 amino acids apart. WW domains interact with PY(PPXY) motifs or phospho-serine/threonine residues in substrate proteins [9]. The HECT domain is comprised of ~350 residues and is responsible for ubiquitin transfer from a conserved cysteine residue to a lysine residue in a substrate protein. Protein structures for C2, WW, and HECT domains have also been solved separately (reviewed in [10]).

Since the original discovery of the *Nedd4* gene, eight related proteins, including Nedd4-2/Nedd4L, WWP1/Tiul1, WWP2, AIP4/Itch, Smurf1, Smurf2, HecW1/NEDL1, and HecW2/NEDL2 (Table 1) have been identified in human and the mouse. Although the similar structure and expression patterns of the Nedd4 family members suggest functionally redundant roles for these proteins, recent studies have begun to define specific functions for individual members. The evolution of Nedd4-like E3s has been described in three excellent review papers [11–13]. Here we mainly focus on their potential roles in cancer.

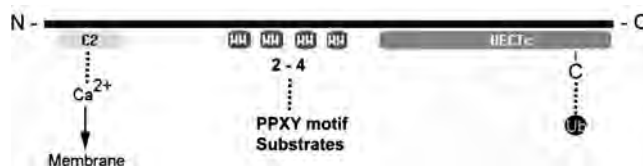


Fig. 1 Schematic diagram showing the structural organization of the nine mammalian Nedd4-like E3 ubiquitin ligases. The C2 domain translocates the protein to the membrane upon calcium binding. The two to four WW domains are known to bind substrate proteins containing PY motifs. The catalytic cysteine within the HECT domain is responsible for ubiquitin transfer

2 Genetic aberrations and alterations in expression of the Nedd4-like E3s in human cancer

To date, the genetic data suggest that several Nedd4-like E3s could function as oncogenic proteins. Smurf2, WWP1, Nedd4, and Smurf1 have all been found to be overexpressed in cancer cells (Table 1).

2.1 Smurf2

Smurf2 has been implicated in suppressing TGF β signaling by targeting Smad2 and TGF β receptor 1 (T β R1) for ubiquitin-mediated degradation [14, 15] (see below in detail). From immunohistochemical staining, Fukuchi et al. found that Smurf2 protein expression in esophageal squamous cell carcinoma correlated with lack of Smad2 phosphorylation, depth of invasion, lymph node metastasis, and survival [16]. Thus, increased Smurf2 expression results in more aggressive esophageal squamous cell carcinoma and a poorer prognosis. While TGF β is known to inhibit epithelial cell proliferation, it also stimulates proliferation of fibroblasts. Consistent with this, when

Smurf2 is overexpressed in fibroblasts, TGF β signaling is inhibited and a senescent phenotype results [17].

2.2 WWP1

The *WWP1* gene maps to 8q21, a region that frequently displays a gain of copy number in human cancers, including prostate and breast cancer. The copy number of *WWP1* was shown to increase in 44–51% of prostate and breast cancer cell lines [18, 19]. Consistent with this observation, the amplification of the *WWP1* gene was confirmed in 31–41% of primary tumors. Although *WWP1* was also mutated in two prostate cancer samples, the frequency is relatively low. In agreement with the reported gene copy number gain, the expression of WWP1 is also upregulated at the mRNA and protein levels in 58–60% of cancer samples from the prostate and breast. The overexpression of WWP1 significantly correlated with the gene copy number gain in both cancer types [18, 19]. These findings strongly suggest that WWP1 gene could be an oncogene in prostate and breast cancer.

Table 1 The Nedd-like E3 ubiquitin ligases and cancer

E3	Gene locus	Alterations in cancer	Substrates	Function	Expression
Nedd4	15q21	Overexpression in prostate and bladder cancer	ENaC, pTEN, IGF-1R, p63, VEGF-R2, Cbl-b, EPS15, Hrs	Hypertension, genome integrity	Kidney, liver, muscle, brain, and heart
Nedd4-2/ Nedd4L	18q21	Alternative splicing in prostate and breast cancer cell lines	ENaC, TrKA, T β R1, Smad2, Smad4, EPS15	Hypertension, TGF β signaling, apoptosis	Kidney, testis, liver, lung, brain, and heart
WWP1/Tiul1	8q21	Amplification and overexpression in prostate and breast cancer, alternative splicing	T β R1, Smad2, 4, KLF2, KLF5, RUNX2 p53, EPS15	TGF β signaling, apoptosis, bone development	Heart, muscle, placenta, kidney, liver, pancreas and testis
WWP2	16q22.1		OCT4, ENaC		Heart, brain, placenta, muscle, and pancreas
AIP4/Itch	20q11.22-q11.23		NF-E2, JunB, c-Jun, p63, p73, RNF11, Cbl, EPS15, Hrs, endophilinA1, CXCR4, Notch, C-FLIP _L , HEF1, Gli	TGF β signaling, apoptosis, immuno cell differentiation	Heart, kidney, brain, lung, spleen, testis, liver, placenta, muscle, and pancreas
Smurf1	7q21.1-q31.1	Amplified and overexpressed in pancreatic cancer	MEKK2, RUNX2, RUNX3, Smad1-5, RhoA	Cell motility, bone development TGF β /BMP signaling	Placenta, pancreas, and testis
Smurf2	17q22-23	Overexpressed in esophageal squamous cell carcinoma	T β R1, SnoN, Smad1, 2, 4, 5, RUNX2, RUNX3, RNF11	TGF β signaling, Senescence, bone development	
HecW1/NEDL1	7p14.1-p13	Differential expression in neuroblastoma	SOD1, Dvl1	Protein quality control, Wnt signaling	
HecW2/NEDL2	2q32.3-q33.1		p73		

Functional analysis of WWP1 further supports the notion that WWP1 promotes prostate and breast epithelial cell proliferation and survival [18, 19]. RNAi mediated *WWP1* knockdown significantly suppressed PC-3, BT474, MCF7, and HCC1500 cancer cell proliferation. In the latter two breast cancer cell lines, WWP1 inhibition caused significant amounts of cell apoptosis [19]. Our results are consistent with two previous reports that shRNA directed against WWP1/Tiul1 (TGIF interacting ubiquitin ligase 1) sensitizes kidney-derived MDCK cells to TGF β -induced growth arrest [8] and WWP1 siRNA decreased cell viability of kidney-derived HEK293T cells [20]. In contrast, forced overexpression of WWP1 in two immortalized breast epithelial cell lines, MCF10A and 184B5, enhances cell proliferation [19]. Interestingly, WWP1 appears to promote cell proliferation in a manner independently of ligase activity [19].

CeWWP1 is essential for *Caenorhabditis elegans* development [21]. However, *Wwp1* knockout mice are viable and fertile with no gross or histological abnormalities, suggesting that there is functional redundancy among members of the Nedd4 family. In support of this hypothesis, the combination of loss-of-function *Wwp1* and *Itch* results in postnatal lethality within 72 h of birth due to lung hemorrhage (Matesic et al., manuscript in preparation). Since *WWP1* is amplified and overexpressed in prostate and breast cancer, tissue specific transgenic mouse models may better mimic the genetic alteration of *WWP1* in human cancer.

Similar to the genetic alteration of *WWP1* in breast and prostate carcinomas, the *Smurf1* gene, mapping to 7q21.1-31.1, was reported to be amplified and overexpressed in pancreatic cancer [22, 23]. Most recently, Nedd4 was specifically shown to be upregulated in invasive bladder cancer and to negatively regulate pTEN activity [24] (See below in detail).

3 The Nedd4-like E3s regulate membrane growth factor receptors

The first substrate identified for Nedd4 was the epithelial sodium channel (ENaC), which plays an important role in hypertension [25]. The regulation of ENaC by Nedd4-like E3s has been studied extensively. *Nedd4-2* null mice develop hypertension due to the lack of ENaC downregulation [26]. A growing body of evidence suggests that Nedd4-like E3s also regulate endocytosis and degradation of multiple growth factor receptors, including insulin-like growth factor 1 receptor (IGF-1R), vascular endothelial growth factor receptor 2 (VEGF-R2), chemokine (C-X-C motif) receptor 4 (CXCR4), Notch, T β R1, epidermal growth factor receptor (EGFR), and the Trk receptor. These growth factor receptors play important roles in tumorigenesis. The deregulation of these receptors results in cell transformation and invasive growth.

It is well established that many growth factor receptors undergo ligand-dependent endocytosis and degradation in lysosomes. This is one of the most important cellular mechanisms to prevent continuous receptor activation in response to growth factor ligands. Receptors are usually recruited to clathrin-coated pits and internalized into the early endosome. Internalized receptors are either recycled back to the plasma membrane or sorted to multi-vesicular bodies (MVB) and subsequently to the lysosome for degradation. It is the ubiquitin moiety that serves as a sorting signal for receptor endocytosis and sorting (reviewed in Hicke and Dunn [3]).

3.1 The Nedd4-like E3s regulate the degradation of multiple membrane receptors

3.1.1 TGF β receptor 1

Smurf1, Smurf2, WWP1, Nedd4-2, and AIP4 have been found to negatively regulate the TGF β signaling pathway (reviewed in Izzi and Attisano [27]). It is well documented that the TGF β signaling pathway regulates epithelial cell proliferation, differentiation, migration, and apoptosis [28]. Transformed epithelial cells usually lose sensitivity to TGF β -induced inhibition. In contrast, TGF β can promote cancer cell invasion and metastasis at late stages. Several Nedd4-like E3s induce ubiquitination and degradation of T β R1, a receptor serine/threonine kinase. Since T β R1 does not have a PY motif, Smad7 was reported to function as an adaptor for Smurf1 [29, 30], Smurf2 [15], Nedd4-2 [31], and WWP1 [32] to recruit T β R1 for ubiquitination. Recently, AIP4 and WWP1 were demonstrated to promote formation of Smad7/T β R1 complex through a ubiquitin-independent mechanism [33]. In parallel, Smurf1 also targets bone morphogenetic protein (BMP) type 1 receptor for degradation [34]. BMP receptors are known to utilize some of the same Smads for signal transduction. These Smads are also targeted by Nedd4-like E3s for ubiquitin-mediated proteasomal degradation (See below in detail).

3.1.2 Notch receptors

AIP4/Itch was documented to negatively regulate the Notch1 receptor. Notch is an important transmembrane receptor that regulates cell fate and oncogenesis. Misregulation of Notch toward either direction is associated with tumor formation [35, 36]. Upon ligand binding, the Notch receptor is cleaved and the soluble cytoplasmic domain translocates into the nucleus to activate gene transcription. Recent observations have demonstrated the importance of Notch endocytosis and degradation by multiple ubiquitin ligases. Itch was first shown to mono- and polyubiquitinate Notch1 [37]. Itch can directly bind to the N-terminal region

of the Notch1 receptor, which has no PY motif [37]. Interestingly, Itch-mediated Notch ubiquitination and degradation can be enhanced by Numb [38]. Further, it was recently discovered that the increased amounts of full length Notch1 found in *itchy* mice can generate an AKT-cell survival signal that contributes to the genesis of autoimmune disease [39]. This increase in full length Notch1 was specific to the activity of Itch, as similar changes in Notch1 levels were not observed in loss-of-function Wwp1 animals (LE Matesic, NG Copeland, and NA Jenkins, unpublished observations). Accumulating evidence from *C. elegans* and *Drosophila* also support the hypothesis that Nedd4-like E3s inhibit Notch signaling. CeWWP1 has been shown to promote Notch degradation but not internalization [40]. In *Drosophila*, dNedd4 and Su(dx) were shown to negatively regulate Notch by directly ubiquitinating the receptor and altering the amount of full length Notch available at the cell surface [41]. In addition to the Notch receptor, Itch was reported to promote ubiquitination and lysosomal degradation of DTX, a RING finger E3 that has been shown to promote Notch signaling in certain cellular contexts [42].

3.1.3 Insulin-like growth factor 1 receptor

Nedd4 was reported to downregulate IGF-1R [43]. IGF-1R has been implicated in the initiation and development of many different human cancers [44]. It is also a promising drug target because many tumor cells undergo apoptosis when the IGF-1R is downregulated [45]. Vecchione and colleagues found that Nedd4 forms a complex with Grb10 and IGF-1R in mouse embryo fibroblasts (MEF). This interaction is mediated by the SH2 domain of Grb10 and the C2 domain of Nedd4 [46]. Interestingly, IGF-1R, but not Grb10, was shown to be ubiquitinated, suggesting that Grb10 serves as an adaptor to mediate the interaction between Nedd4 and IGF-1R. In support of this notion, overexpression of a catalytically inactive form of Nedd4 decreased IGF-1R ubiquitination and degradation upon IGF-1 stimulation [43].

3.1.4 Vascular endothelial growth factor receptor 2

Nedd4 was also demonstrated to downregulate VEGF-R2 [47]. VEGF is an important angiogenic factor which stimulates cell proliferation, migration, and angiogenesis [48]. Binding of VEGF to the tyrosine kinase receptor VEGF-R2 induces receptor dimerization, autophosphorylation, and signaling. VEGF-R2 is also ubiquitinated in response to VEGF. Overexpression of Nedd4 was shown to promote VEGF-R2 degradation [47]. Murdaca et al., proposed that Nedd4 indirectly regulates the ubiquitination of VEGF-R2 in response to VEGF because ectopic over-

expression of a catalytically inactive form of Nedd4 does not inhibit VEGF-R2 ubiquitination [47]. Interestingly, Grb10 was confirmed to interact with Nedd4 in this study. In contrast to the role of Grb10 in the ubiquitination of IGF-1R by Nedd4, overexpression of Grb10 suppressed the degradation of VEGF-R2 by Nedd4. However, whether Nedd4 regulates IGF-1R and VEGF-R2 degradation under physiological conditions remains to be determined.

3.1.5 Chemokine (C-X-C motif) receptor 4

AIP4 was shown to downregulate CXCR4 [49]. Binding of stromal cell-derived factor-1 (SDF-1) to the G protein coupled receptor CXCR4 activates a variety of intracellular signal transduction pathways that promote cell survival, proliferation, chemotaxis, migration and adhesion [50]. Overexpression of CXCR4 in tumor cells is strongly associated with increased metastatic potential [51]. AIP4, but not Nedd4 and Nedd4-2, specifically promotes SDF-1 induced CXCR4 mUb and lysosomal degradation [49]. It was proposed that AIP4 indirectly regulates CXCR4 through hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and Vps4 [49].

3.1.6 Epithelial growth factor receptors

There is some data suggesting that Nedd4-like E3s can regulate EGFRs, although these data are far from conclusive. It is well established that the abnormal activation of the EGF pathway is a common theme in epithelial cancer [52]. Although genetic amplification and mutation are involved in over-activation of the EGFR family, misregulation of the degradation of this family also contributes to cancer development. It has been shown that Nedd4 inhibits EGF-independent EGFR endocytosis and degradation through its antagonization of Cbl-b, a RING E3 that normally functions to monoubiquitinate the EGFR and target it for turn over by the lysosome [53]. It has also been proposed that Nedd4 regulates ligand-independent EGFR degradation by ubiquitinating Hrs [54]. Thus, by these mechanisms, Nedd4 effectively increases the amount of EGFR available at the cell surface, and lowers the required signaling threshold. AIP4/Itch has also been shown to interact with Cbl-c [53, 55]. Angers et al., found that Itch regulates EGFR by ubiquitinating endophilin A1 [56]. In contrast, Marchese and colleagues observed that AIP4 siRNA has no effect on EGF induced EGFR endocytosis and degradation in HeLa cells [49]. Most recently, Itch was shown to target ErbB4 for degradation [57].

In addition to the receptors noted above, nerve growth factor (NGF)-dependent TrkA receptor [58] was reported to be a substrate of Nedd4-2. Nedd4-2 was demonstrated to constitutively bind to an unphosphorylated PY motif on

TrkA through its WW domains. Interestingly, Nedd4–2 overexpression caused apoptosis of NGF-dependent neurons.

The data reviewed here indicate that a number of receptors or membrane proteins are regulated by Nedd4-like E3s. Some of these results require further validation since the overexpression of Nedd4-like E3s can induce profound consequences on the intracellular trafficking machinery (see below). It is therefore essential that the results obtained from such experiments be validated with RNAi and *in vivo* approaches. Further, there is the consideration of how the Nedd4-like E3 function in a given cellular context. Signaling pathways targeted by the Nedd4-like E3s, including the TGF β and Notch pathways, play context-dependent roles in tumorigenesis. Therefore, the action of an individual Nedd4-like E3 in cancer could also be context-dependent.

3.2 The Nedd4-like E3s regulate the endocytosis machinery

The molecular determinants mediating the recruitment of various Nedd4-like E3 to different membrane receptors are not clear. Some Nedd4-like E3s have been demonstrated to directly ubiquitinate growth factor receptors. However, under normal conditions, Nedd4-like E3s do not directly interact with these receptors. Nedd4-like E3s have been shown to ubiquitinate multiple members of the endocytosis machinery such as Cbls, Endophilin A1, epithelial growth factor receptor substrate 15 (EPS15), Hrs, and N4WBPs.

3.2.1 Cbls

The RING finger E3 ligases Cbl-b and Cbl-c are regulated by Nedd4 and AIP4 [53, 55]. The endocytosis and degradation of EGFR have been extensively studied. Binding of EGF to EGFR results in dimerization and autophosphorylation of several tyrosine residues in the cytoplasmic tail of the EGFR. The phosphorylation of EGFR at Tyr1045 provides a binding site for the SH2 domain of the Cbl E3 ligases [59]. Subsequently, Cbls are phosphorylated and activated to monoubiquitinate the EGFR. This step was proved to be necessary and sufficient for EGFR endocytosis and degradation. Additionally, Cbls are negative regulators of several receptor tyrosine kinases such as PDGFR [60] and c-Met [61]. Nedd4 was reported to directly ubiquitinate Cbl-b and target it for proteasomal degradation. This has the effect of concomitantly blocking Cbl-b mediated EGFR endocytosis and degradation [53].

3.2.2 Endophilin

The endophilin-Cbl-interacting protein of 85 KDa (CIN85)-Cbl complex is required for ligand-stimulated receptor

internalization [61, 62]. Angers et al. found that AIP4 interacts with and ubiquitinates Endophilin A1 at the endosome upon EGF treatment [56]. This finding is also supported by a study in yeast. Rvs167p and Sla1p, orthologues of the mammalian endophilin and CIN85 proteins, were shown to be ubiquitinated by Rsp5p (the only ortholog of the mammalian Nedd4-like E3s in yeast *Saccharomyces cerevisiae*) [63]. Ubiquitination of Endophilin and CIN85 may affect rapid EGFR internalization because these proteins play an important role in regulating clathrin-coated vesicle budding.

3.2.3 Epithelial growth factor receptor substrate 15

EPS15 is regulated by multiple Nedd4-like E3s. It has been reported that ubiquitinated EGFR can be recognized by the ubiquitin-binding protein EPS15 or by Epsin, which promotes EGFR internalization to the early endosome [64]. Interestingly, EPS15 and Epsin undergo mUb mediated by their own ubiquitin interaction motif (UIM) [65, 66]. Recently, mUb was proposed to inactivate the function of EPS15 since the intermolecular interaction between ubiquitin and the UIM supplants the association between the ubiquitinated receptor and the UIM [67]. Nedd4, Nedd4–2, AIP4, and WWP1, but not WWP2, were reported to ubiquitinate EPS15 [66]. We found that WT WWP1, but not the C890A mutant, specifically increased EPS15 ubiquitination (Fig. 2). EPS15 does not contain a PY motif. Instead, it was proposed that the ubiquitination of an E3 ligase itself provides a platform for EPS15 binding to the E3 through their UIM domain [66]. Most recently, the E3 ligase Parkin was reported to mono-ubiquitinate EPS15 and delay EGFR degradation [68]. The Nedd4-family of E3s may also decrease EGFR endocytosis through a similar mechanism, although this hypothesis awaits more direct experimental proof.

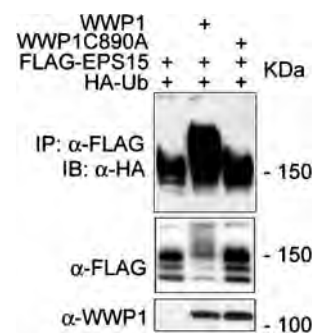


Fig. 2 WWP1 ubiquitinates EPS15. WT or catalytic inactive WWP1C890A were co-transfected with FLAG tagged EPS15 (kindly provided by Dr. Ivan Dikic) and HA tagged Ub into HEK293T-derived LinX cells in 60-mm plates. Cell lysates were immunoprecipitated with FLAG-M2 beads (Sigma) under denaturing conditions and detected using the anti-HA antibody. Five percent of the cell lysate used in the immunoprecipitation was loaded on a gel for Western blot analysis with anti-FLAG and anti-WWP1 antibodies

3.2.4 Hepatocyte growth factor-regulated tyrosine kinase substrate

In addition to the Cbls, Endophilin, and EPS15, Nedd4 and AIP4 have also been shown to ubiquitinate Hrs, another ubiquitin-binding protein [49, 54]. Upon delivery to early endosome, the EGFR is either recycled back to the plasma membrane or sorted to MVB and then the lysosome. Hrs was found to promote the sorting of EGFR and CXCR4 to the lysosome for degradation [49, 54]. Similar to EPS15, mUb of Hrs by Nedd4 or AIP4 may cause the loss of Hrs endocytic activity by the intramolecular interaction between the ubiquitin and UIM. Interestingly, yeast two hybrid analysis demonstrated that Hrs does not interact with WWP1 and WWP2 [20], indicating a specificity in this effect.

3.2.5 N4WBPs

Furthermore, Nedd4-like E3s may regulate several other proteins in the MVB or Golgi network such as N4WBP4/PMEPA1 [69], N4WBP5 (Ndfip1) [70], and N4WBP5A (Ndfip2) [71]. All of these Nedd4-interacting proteins contain PY motifs. Nedd4, Nedd4–2, WWP2, AIP4, but not Smurf1, were shown to interact with N4WBP5A [71]. N4WBP5A protein was shown to regulate EGFR endocytosis in HeLa cells [71]. Additionally, Nedd4 and Nedd4–2 ubiquitinate N4WBP5A but do not target N4WBP5A for degradation. Interestingly, the expression of N4WBP4/PMEPA1 is induced by androgen and downregulated in prostate cancer, and its tumor suppressor function requires the PY1 motif [69]. It was proposed that PMEPA1 serves as an adaptor that allows Nedd4 to recruit androgen receptor (AR) for ubiquitination and degradation [69]. This negative regulatory loop may be disrupted in prostate cancer due to the loss of expression of PMEPA1 [72]. Whether Nedd4-like E3s regulate receptors by ubiquitinating N4WBP proteins under physiological conditions needs to be investigated further.

AIP4 and Smurf2 were found to interact with the RING finger E3 ligase RNF11 [73, 74]. RNF11 has also been found to localize to MVB and to promote EGFR endocytosis and degradation [75]. Thus, we speculate that Nedd4-like E3s regulate receptor trafficking via multiple mechanisms by targeting proteins involved in endocytosis such as Cbls, EPS15, Hrs, N4WBP5, or RNF11. Importantly, these lines of evidence imply that Nedd4-like E3s upregulate EGFR. However, direct experimental evidence in support of this hypothesis is still lacking. Regardless of the mechanism, it is well documented that impaired downregulation of growth factor receptors is strongly associated with cancer [76]. It will be interesting to see whether Nedd4-like E3s promote cancer development by regulating the degradation of multiple growth factor receptors.

4 Regulation of cancer-related transcription factors

Multiple cancer-related transcription factors are regulated by the Nedd4-like E3s through ubiquitin-mediated degradation and relocalization. Since transcription factors regulate the transcription of numerous genes, the abnormal regulation of those same factors plays an important role in tumorigenesis. The first hint that Nedd4-like E3s regulate transcription factors came from the observation that the localization of these E3s is shuttling from the cytoplasm to the nucleus. In recent years, Nedd4-like E3s have been demonstrated to regulate multiple transcription factor families, such as Smad, p53, krüppel-like factor (KLF), RUNX, and Jun.

4.1 Smad family

It is well established that Smad proteins play crucial roles in the TGF β and BMP signaling pathways. The activated TGF β receptors phosphorylate Smad2 and Smad3. Then, phosphorylated Smad2 and Smad3 form complexes with Smad4 and translocate into the nucleus to regulate gene transcription. The TGF β pathway plays an important role in tumor development. Consistent with this hypothesis, Smad4 is frequently inactivated in a variety of cancer types [77].

Nedd4-like E3s target several Smad proteins for ubiquitin-mediated degradation. All Smads except for Smad4 and Smad8 contain a PY motif. Not surprisingly, Smurf1, Smurf2, and WWP1 directly interact with all Smads (except Smad4 and Smad8) with slightly different affinities [8, 32]. Smurf1 and Smurf2 have been reported to target Smad1, Smad2, and Smad5 for degradation [14, 78–81]. WWP1 has also been demonstrated to promote Smad2 ubiquitin-mediated degradation [8, 82]. Komuro *et al.* found that WWP1 can ubiquitinate Smad6 and Smad7 but not Smad2 [32]. In fact, Smad2 ubiquitination by WWP1/Tiul1 requires TGIF and TGF β signaling [8]. Thus, Nedd4-like E3s strongly inhibit the TGF β and BMP signaling pathways by targeting Smads for degradation.

Several Smad proteins have been reported to serve as adaptors for Nedd4-like E3s in the targeting of substrates for ubiquitination. For example, Komuro and colleagues demonstrated that Smad7 helps WWP1 and Smurf1 recruit T β R1 [8, 32]. However, whether the Smad7 adaptor protein is ubiquitinated by WWP1 or not is controversial [8, 32]. Smad7 was also demonstrated to function as an adaptor for Smurf2 in targeting β -catenin for degradation [83]. Similar to what has been observed with Smad7, Moren *et al.* found that Smad2 functions as an adaptor for WWP1 in the ubiquitination of Smad4 [8, 84]. The use of Smads as adaptors has also been noted with other members of the Nedd4 family of E3 ligases. For instance, Smurf2 uses Smad2 as an adaptor to target SnoN [85]. Similarly, Smurf1

and WWP1 use Smad6 as an adaptor to target RUNX2 for degradation [86]. Itch uses Smad3 to target Cas¹³⁰ family protein HEF1 for degradation [87]. The usage of adaptor proteins makes the regulation of the TGF β pathway more fine-tuned and also expands the repertoire of substrates for Nedd4-like E3s.

4.2 p53 family

The p53 transcription factor is the most extensively studied tumor suppressor in human cancer. p53 activation up-regulates growth arrest- and apoptosis-related genes in response to stress signals, thereby leading to either cell cycle arrest, senescence, or apoptosis [88]. Two p53 homologues, p63 and p73, have overlapping and distinct functions in the regulation of gene expression [89]. Indeed, both p73 and p53 can be induced by DNA damage and activate some common genes to suppress growth or induce apoptosis. Accumulating evidence supports the idea that the full-length transactivation (TA) isoforms (i.e., those containing the TA domain) of p63 and p73 have pro-apoptotic properties, whereas the Δ N isoforms of p63 and p73 generally have anti-apoptotic properties [90]. The Δ N isoforms of p63 and p73 have also been shown to suppress cell proliferation [91, 92]. The p53 family of transcription factors are tightly regulated by multiple mechanisms including ubiquitin-mediated degradation and subcellular relocalization [90, 93, 94].

WWP1 was shown to ubiquitinate p53, the major form being mUb [95]. It is well established that Mdm2 can mono-ubiquitinate p53 which results in the exportation of nuclear p53 to the cytoplasm [96]. Nuclear export of p53 can also be mediated by WWP1 [95]. Consequently, p53 transcriptional activity is inhibited by WWP1, despite elevations in the p53 protein level [95].

The Nedd4-like E3 NEDL2/HecW2 was identified as the first E3 that binds and ubiquitinates p73 via a WW/PY interaction [97]. However, NEDL2 does not bind to p53, which lacks the PY motif. NEDL2 has been shown to stabilize p73 and increase its transcriptional activity although the precise mechanism by which this occurs remains unclear [97]. It was also reported that Itch targets p73 for ubiquitin-mediated proteasomal degradation [98, 99]. In contrast to NEDL2, Itch negatively regulates the transcriptional activity of p73 [98]. It was proposed that Itch is rapidly downregulated upon DNA damage, allowing the TA-p73 protein to accumulate and induce cell growth arrest and apoptosis [98]. Recently, YAP1 was demonstrated to stabilize p73 by displacing Itch binding to p73 [100].

In addition to p53 and p73, p63 activity has also been shown to be modulated by Nedd4 [101] and Itch [102–104]. Itch interacts with TA-p63 and Δ Np63 via a WW/PY

interaction and targets them for ubiquitin-mediated proteasomal degradation [103]. However, whether Nedd4-like E3s modulate tumor development through the regulation of the abundance of various p63 and p73 isoforms has not yet been determined.

4.3 KLF family

KLF is a transcription factor family which consists of over 20 members in humans, and is structurally characterized by three tandem zinc-finger domains at the C-terminus [105, 106]. The members of this family form a network that regulates a diverse range of biological processes, including cellular proliferation, cell cycle, apoptosis, differentiation, and angiogenesis [105, 106]. Several members of the KLF family, such as KLF2 [107] and KLF5 [108], have been implicated in the development of a variety of human cancers.

In 2001, work from Jerry Lingrel's laboratory first suggested that WWP1 inhibits the transcriptional activity of KLF2 [109]. KLF2 inhibits cell growth [107], angiogenesis [110], and sensitizes cells to DNA damage-induced apoptosis [111]. Further investigation revealed that WWP1 targets KLF2 for ubiquitin-mediated proteasomal degradation. The inhibitory domain of KLF2 is essential for interacting with WWP1 because KLF2 has no PY motif. Surprisingly, WWP1 promotes KLF2 ubiquitination independent of E3 ligase activity and may serve as an adaptor to recruit another E3 ligase.

Recently, KLF5 was demonstrated to be a substrate of WWP1. KLF5 is a key regulator of embryonic development, tissue remodeling, angiogenesis, adipocyte differentiation, epidermal development and tumorigenesis. The KLF5 protein turns over rapidly via the ubiquitin-proteasome pathway [112]. This is mediated by WWP1, which specifically interacts with KLF5 via the PY motif of KLF5 and targets KLF5 for proteasomal degradation [113]. Interestingly, the expression of WWP1 is negatively correlated with KLF5 protein expression in human prostate and breast cancer. Although the exact role of KLF5 in cancer remains to be elucidated, these findings suggest that WWP1 may contribute to tumorigenesis through promoting KLF5 ubiquitination and degradation.

4.4 RUNX family

The three mammalian Runt homology domain transcription factors (RUNX1, RUNX2, RUNX3) control genes involved in the differentiation of distinct tissues. The RUNX family can also function as cell context-dependent tumor suppressors or oncogenes. Both RUNX2 and RUNX3 contain conserved PY motifs.

RUNX2 is a bone-specific transcription factor that functions in regulating bone development and metastasis

[114]. RUNX2 is frequently overexpressed in invasive breast and prostate cancer [114]. Smurf1, Smurf2, and WWP1 were reported to target RUNX2 for ubiquitin-mediated degradation via either WW/PY motifs or a Smad6 adaptor [86, 115, 116]. WWP1 was demonstrated to regulate RUNX2 *in vivo* [117]. Another adaptor protein Schnurri-3 was shown to enhance Runx2 ubiquitination by Wwp1 [117]. Transgenic mice that express Smurf1 specifically in osteoblasts show reduced bone formation during postnatal life due to a decrease in Runx2 [118]. However, Runx2 is not upregulated in Smurf1 null osteoblasts *in vivo* [119].

In contrast to RUNX2, *RUNX3* functions as a gastric tumor suppressor. The expression of *RUNX3* is lost in about 60% of primary gastric cancer specimens [120]. Both Smurf1 and Smurf2 have been demonstrated to promote RUNX3 ubiquitination and degradation [121]. Interestingly, p300 mediated RUNX3 acetylation protects RUNX3 from ubiquitin-mediated degradation [121].

4.5 Jun family

JunB and c-Jun were identified as direct targets of Itch [122, 123]. JunB has been shown to play an important role in T cell differentiation [124] while c-Jun may function in T cell activation [125]. The regulation of JunB and c-Jun by Itch is further refined by phosphorylation (detailed later) and by interaction with other proteins. Itch has recently been shown to immunoprecipitate with *N4WPBP5/Ndfip1* and to co-localize in T cells [126]. *Ndfip1*^{-/-} mice have a similar phenotype to *itchy* mice, including severe inflammation and a Th2 bias in T cell differentiation. Further, levels of JunB are increased in *Ndfip1*^{-/-} T cells, suggesting that *Ndfip1* is required for efficient ubiquitination of JunB by Itch. Since *Ndfip1* is a membrane-bound protein, it may act to recruit Itch to the appropriate subcellular compartment for Itch to exert its effects.

As targets of the TGFβ pathway [127], JunB and PAI1 are inhibited by WWP1/Tiul1 in MDCK cells through an indirect mechanism [8]. Work from the Zhang lab has demonstrated that JunB is upregulated in Smurf1 null osteoblasts [119]. However, an upstream kinase of JunB, MEKK2, was identified as the authentic, direct physiological target of Smurf1 but not Smurf2. MEKK2 interacts with Smurf1 via the PY motif of MEKK2 and first two WW domains of Smurf1. Interestingly, the auto-phosphorylation of MEKK2 is a prerequisite for Smurf1 mediated MEKK2 ubiquitination. Consistent with these observations, Smurf1-deficient mice exhibit an age-dependent increase of bone mass [119].

NF-E2 is a hematopoietic transcription factor containing two subunits. The large subunit p45 is homologous to c-Jun and bears PY motifs. The p45 protein was found to bind to

WWP1 and Nedd4 [128]. Yeast two hybrid experiments showed that p45 could interact with Itch. Further analysis demonstrated that Itch might function as a transcriptional co-repressor of NF-E2 [129].

Additionally, Nedd4-like E3s may target other transcription factors for degradation. For example, Itch has been shown to ubiquitinate and degrade the transcription factor Gli1, which plays an important role in Hedgehog signaling in cell development and tumorigenesis [130]. As with the regulation of Notch, Numb can promote Itch-dependent Gli1 ubiquitination and degradation. Oct-4 is an important transcription factor that affects the fate of mammalian embryonic stem cells. WWP2 has been shown to promote Oct-4 ubiquitin-mediated proteasomal degradation [131]. Finally, Nedd4/hRPF1 has been shown to potentiate the transcriptional activity of nuclear receptors including progesterone receptor and glucocorticoid receptor [132].

5 Other substrates of the Nedd4-like E3s in cancer

5.1 Nedd4 and pTEN

pTEN is an important tumor suppressor protein in various types of cancers. The pTEN phosphatase negatively regulates PI3K/Akt signaling [133], which is critical for cancer cell survival. Additionally, nuclear localization of pTEN has been proposed to maintain genome stability and tumor suppression [134]. pTEN is frequently inactivated by gene deletion or mutation [135]. Intriguingly, pTEN is also ubiquitinated and degraded through the proteasome although it is a relatively stable protein [136]. Recently Nedd4 was identified as a specific E3 ligase for pTEN. Nedd4 not only targets pTEN for proteasomal degradation through polyubiquitination but also transports pTEN into the nucleus through mUb [137].

By decreasing the level of pTEN protein, Nedd4 was shown to promote Akt signaling [24]. Overexpression of Nedd4 collaborates with K-Ras to transform p53 deficient MEF. Additionally, *Nedd4* RNAi inhibited DU145 (pTEN positive) but not PC-3 (pTEN negative) xenograft growth. Consistently, the mRNA level of Nedd4 was shown to be upregulated in invasive bladder cancer samples [24]. Taken together, Nedd4 was proposed to be an oncoprotein and a potential target for pharmacological intervention in pTEN positive tumors.

5.2 HecW1/NEDL-1 and dishevelled-1

Miyazaki et al. identified a novel, differentially expressed Nedd4-like E3, NEDL1, in brain tumors [138]. *NEDL1* mRNA is preferentially expressed in neuronal tissue and is

highly expressed in neuroblastomas with favorable prognosis. Through yeast-two hybrid screening, NEDL1 was demonstrated to be associated with two proteins, dishevelled-1 (Dvl1) and translocon-associated protein δ (TRAP- δ), which bind to mutant but not WT superoxide dismutases-1 (SOD1). As a result, NEDL-1 targets mutant Dvl1 and SOD-1, but not TRAP- δ , for ubiquitination and degradation. Because TRAP- δ localizes to the ER, NEDL-1 was proposed to regulate protein quality control through an ER-associated degradation pathway [138]. Interestingly, Dvl1 is one of the key transducers in the Wnt/ β -catenin signaling pathway. Thus, NEDL-1 may play a role in motor neuron differentiation and apoptosis through its regulation of Dvl1 degradation [138]. The role of NEDL1 in brain or other types of tumors remains to be elucidated.

5.3 Itch and apoptosis

Itch was demonstrated to target long FLICE (pro-caspase-8)-inhibitory protein (c-FLIP_L) for ubiquitin-mediated proteasomal degradation [139]. FLIP can be upregulated by NF- κ B and specifically inhibits caspase-8 activation and tumor necrosis factor- α (TNF α) induced apoptosis [140]. Chang et al. found that c-FLIP_L ubiquitination by Itch is regulated by JNK mediated Itch phosphorylation [139]. However, the work fails to identify the domain of Itch that interacts with the CASP domain of c-FLIP_L. This is important because c-FLIP_L does not have a PY motif, yet no other Nedd4-like E3s have been reported to have similar function on c-FLIP_L turnover.

Loss of function *Itch* (*itchy*) mice display a variety of immunological and inflammatory disorders as they age, including inflammation of the lung and stomach and hyperplasia of lymphoid and hematopoietic cells [141]. It was noticed that the *itchy* mice have a persistent itching behavior. Part of this can be attributed to the Th2 bias in differentiation of T cells from animals, which results in an allergic response with increased IgG1 and IgE levels in the serum, as well as eosinophil activation [122]. Additionally, it was reported that *itchy* mice have increased levels of full length Notch1 which may complex with PI3K and p56^{lck} in T cells. This results in increased amounts of phospho-AKT and decreased apoptosis in developing thymocytes [39]. These data speak to the fact that Itch likely plays a physiological role in mediating apoptosis through a variety of different pathways under the influence of a number of molecular signals.

5.4 Smurf1, RhoA, and invasion

Accumulating evidence points to the fact that Smurf1, but not Smurf2, promotes RhoA ubiquitination and degradation and regulates cell motility [142–145]. RhoA, Rac, and

Cdc42 proteins belong to Ras superfamily of GTPases. RhoA binds and hydrolyzes the conversion of GTP to GDP. The GTP-bound form of RhoA is active. The active form regulates cell morphology, differentiation, migration, and division. Smurf1-mediated RhoA degradation is crucial for TGF β -induced dissolution of tight junctions during epithelial-mesenchymal transition [143]. Boyer et al. demonstrated that the induced degradation of RhoA is blocked in Smurf1 null MEF cells and Smurf1 selectively ubiquitinates GTP-bound active RhoA [144]. However, total RhoA protein levels are not increased in Smurf1 knockout mice and Smurf1 siRNA transfected cells [119, 145]. Sahai et al. demonstrated that Smurf1-mediated RhoA degradation only occurs at the cell periphery [145]. Furthermore, Smurf1 inhibition in cancer cells increases cell motility and favors intravasation, but is not sufficient to promote metastasis *in vivo* [145].

5.5 Nedd4, Cdc25, and the cell cycle

The Pub1 E3 ligase from *Schizosaccharomyces pombe* is one of the three orthologues of mammalian Nedd4-like E3s. Pub1p was demonstrated to target Cdc25 for ubiquitin-mediated degradation [146]. Cdc25 (Cdc25A, Cdc25B and Cdc25C in mammals) is a key phosphatase regulating the cell cycle through its activation of the Cdks (reviewed in [147]). Cdc25A is ubiquitinated and degraded by the E3 ligase APC/C^{Cdh1} and possible other Skp1-Cul1-F-box E3 complexes [148]. Interestingly, Cdc25A contains a PY motif; and Cdc25C has been demonstrated to bind to the second WW domain of Nedd4 in HeLa cells [9]. It will be interesting to further investigate whether Nedd4 or other family members target Cdc25 for degradation since Cdc25 is frequently overexpressed in human cancer.

6 Ligase independent function of the Nedd4-like E3s

Although the ligase activity of Nedd4-like E3s is very important for their cellular functions, several lines of evidence suggest that Nedd4-like E3s may have ligase-independent functions as well. Overexpression of various Nedd4-like E3-derived WW domains strongly inhibits viral budding, a process requiring protein trafficking [20]. Itch and WWP1 can inhibit TGF β signaling independently of their ligase activities [33]. Smurf2 overexpression has been shown to arrest fibroblasts in a ligase- and C2 domain-independent manner [17]. Recently, we found that catalytically inactive WWP1 promotes breast epithelial cell proliferation as efficiently as the WT WWP1 [19]. These findings suggest that Nedd4-like E3s could regulate trafficking and other cellular processes independently of their ligase activities.

Because E3 ligase activity appears not to be necessary for the function of Nedd4-like E3s under some conditions, it is tempting to speculate that these E3s could affect the action of their partners through just binding. Several WW domain-containing proteins, such as WWOX and YAP, function by binding to PY motif-containing substrates [149]. Zhang et al. mapped the Smurf2 domains required for causing fibroblast senescence and found that although both the WW domains and the HECT domain contribute to Smurf2's senescence-inducing function, the E3 ligase activity is not required.

7 Regulation of the Nedd4-like E3s

7.1 Phosphorylation

AIP4/Itch has been shown to be phosphorylated upon EGFR [55] and IGF1R activation [46]. Additionally, JNK was shown to phosphorylate the proline-rich motif of AIP4/Itch which is active toward JunB, c-Jun, and c-FLIP_L [123, 139]. In contrast, the phosphorylation at Y371 of AIP4 by Fyn significantly reduced the ability of AIP4 to ubiquitinate JunB [150]. An auto-inhibition model between the WW domain and HECT domain was proposed to explain this interesting phenomenon [151]. Recently, Nedd4 was reported to be phosphorylated by Src which is essential for EGF-induced mUb of EPS15 [66].

Nedd4–2 was shown to be serine/threonine phosphorylated in response to several different stimuli, such as insulin, insulin growth factor, aldosterone, vasopressin and NGF [58, 152, 153]. Nedd4–2 is phosphorylated by serum and glucocorticoid-inducible kinase (SGK1), a target of the PI3K/PDK1 pathway. This modification decreases the ligase activity toward ENaC since 14-3-3 will bind to phosphorylated Nedd4–2 and block substrate recognition. Nedd4–2 phosphorylation can also be mediated by vasopressin-activated PKA [152]. Similarly, cytokine independent survival kinase 1 (CISK1), another target of the PI3K/PDK1 pathway, phosphorylates AIP4 and blocks AIP4-mediated CXCR4 degradation induced by SDF-1 [154]. Both SGK1 and CISK1 contain a PY motif, which is

essential for their interaction with Nedd4–2 or AIP4, respectively [154]. Interestingly, the SGK1/CISK1 phosphorylation sites on Nedd4–2 or AIP4 have been identified and are located in the WW domains [154]. Similar mechanisms may exist for other Nedd4-like E3s.

7.2 Ubiquitination and degradation

Itch was found to be rapidly degraded in primary T cells in response to CD3 and CD28 stimulation [122]. Itch degradation may be mediated by self-ubiquitination and enhanced by JNK-mediated phosphorylation [123]. Similarly, we found that the proteasome inhibitor MG132 significantly increased WWP1 protein levels in the MCF10A breast epithelial cell line (Fig. 3(a)). Further, the degradation of WWP1 was almost completely blocked by MG132 in 22Rv1 prostate cancer cells (Fig. 3(b)). As assayed by cycloheximide chase, the half-life of WWP1 is about 3 h in 22Rv1. Other Nedd4-like E3s may be subject to similar autoregulation.

Besides ubiquitin-mediated proteasomal degradation, Nedd4-like E3s may be degraded by cleavage. For example, Nedd4 was shown to be cleaved by multiple caspases during apoptosis [155]. However, the significance of this observation is not known.

7.3 Subcellular localization

Nedd4-like E3s have been found to localize to the plasma membrane, the cytoplasm, and the nucleus. Nedd4-like E3 proteins have been observed on multiple components of the endocytic pathway including the plasma membrane, the early and late endosome, and the Golgi. For example, AIP4 has been reported to localize to various endosomal subdomains [49, 56]. The C2 domain is necessary but not sufficient for endosomal localization. Wwp1 has been detected on early endosomes in the murine skeletal muscle cell line C2C12 [156]. Interestingly, Nedd4 is translocated to mitochondria upon IGF-1R activation, raising the possibility that Nedd4 may mediate the anti-apoptotic signaling of the IGF-1R [157]. Finally, the Nedd4-like proteins have also been localized to the nucleus [20, 113]. A Rev-like nuclear export signal has been identified in Nedd4 [158] and Smurf1 [159].

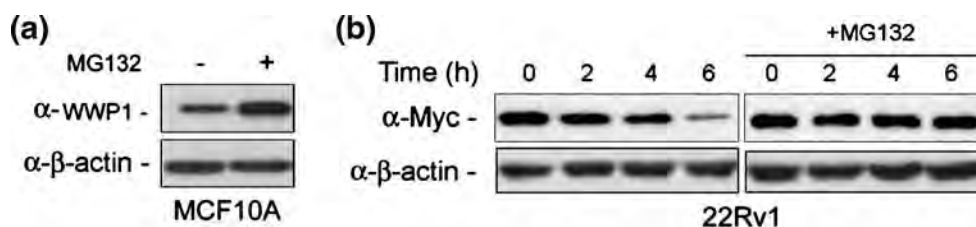


Fig. 3 WWP1 degradation by the proteasome. (a) The MCF10A cell line stably expressing WWP1 was treated with 10 μ M of the proteasome inhibitor MG132 for 12 h. WWP1 protein was detected by Western blot. β -actin was used as a loading control. (b) Myc tagged

mouse Wwp1 was transfected into the 22Rv1 prostate cancer cell line. The half-life of Wwp1 was determined in the absence or presence of 10 μ M MG132 by cycloheximide (CHX) chase assay. One hundred micrograms per milliliter CHX was used in this experiment

Different subcellular locations of Nedd4-like E3s may be dependent upon which proteins the ligases interact with. For example, upon TGF β stimulation, Smad7 accumulates in the nucleus where it interacts with Smurf2 or WWP1. These complexes then translocate out of the nuclei and interact with T β R1 [29, 32]. Similarly, Notch was reported to exclude WWP1 out of the nucleus and instead localize to the early endosome [156]. The HECT domain is necessary and sufficient for WWP1 nuclear localization and regulation by Notch. After inducing C2C12 cell differentiation by serum depletion, nuclear WWP1 relocated to the cytoplasm [156]. These findings highlight the fact that changing subcellular localization is an important way to control the accessibility and activity of Nedd4-like E3s toward different substrates.

7.4 Transcription

Nedd4-like E3s are usually ubiquitously expressed in multiple tissues but some show distinct tissue distributions (Table 1). *Nedd4* is widely expressed in mouse kidney, liver, muscle, brain, and heart [6]. *Nedd4-2* was shown to be expressed in rat testis, kidney, liver, lung, brain, and heart [160]. *Smurf1* is mainly expressed in human placenta, pancreas, and testis [32]. *WWP2* and *AIP4* mRNA are widely expressed in human heart, brain, placenta, muscle, and pancreas [161]. *WWP1* mRNA is strongly or modestly expressed in human heart, muscle, placenta, kidney, liver, pancreas and testis [32, 128]. The expression pattern of *Wwp1* and *Itch* mRNA seems to be conserved in mouse [21, 141]. Interestingly, multiple *Wwp1* isoforms were observed in several mouse tissues.

RNA splicing may be another way to modulate the activity of Nedd4-like E3s. In a breast cancer cell line, T47D, six isoforms of *WWP1* have been identified [162]. Expression of *WWP1* splice variants was detected in multiple tissues and the ratios among the *WWP1* isoforms showed tissue-specific distribution. This observation is reinforced by our recent discovery that *WWP1* mRNA with different molecular sizes was detected in several prostate and breast cancer cell lines by Northern blot and Western blot analysis [18, 19]. Different splice variants of *AIP4* and *Nedd4-2* have also been reported [162, 163], suggesting that RNA splicing could be a common regulatory mechanism for Nedd4-like E3s.

The transcripts of Nedd4-like E3s are responsive to multiple growth factor signals. The mRNA expression of *Smurf1* and *Smurf2* was demonstrated to be induced by TGF β and BMP [164]. Similarly, we found that *WWP1* is upregulated by TGF β in HaCaT and PC-3 cells in a dosage and time dependent manner (Fig. 4). As mentioned earlier, Smurfs and WWP1 are potent inhibitors of TGF β signaling; therefore, this feedback control loop aids in precisely regulating TGF β signal transduction. Further, *Smurf1/2* and

Nedd4 can be induced by the pro-inflammatory factor TNF α [116, 165]. In fibroblasts, *Smurf2* mRNA is upregulated when cells undergo senescence [17].

Several mRNA isoforms of *Nedd4-2* were upregulated by androgen in the AR positive LNCaP prostate cancer cell line [163]. Interestingly, *WWP1* was also reported to be upregulated by androgen and is highly expressed in LNCaP cells [18, 166]. In parallel, a high level of *WWP1* expression is more frequently detected in ER positive breast cancer cell lines [19] and tumors (Oncomine.org). Additionally, progesterone receptor and Glucocorticoid receptor activity are potentiated by Nedd4/hRPF1 [132]. Thus, it is tempting to speculate that Nedd4-like E3s and hormone signaling are mutually regulated.

Upon DNA damage, levels of the endogenous Itch protein were shown to significantly decrease, allowing the TA-p73 protein to accumulate and induce cell cycle arrest and apoptosis [98]. Similarly, WWP1 is also decreased in response to γ -irradiation in p53 WT, but not p53 null, MEF cells [95]. These findings suggest that the transcription of Nedd4-like E3s can be regulated by various DNA damage-response signaling cascades.

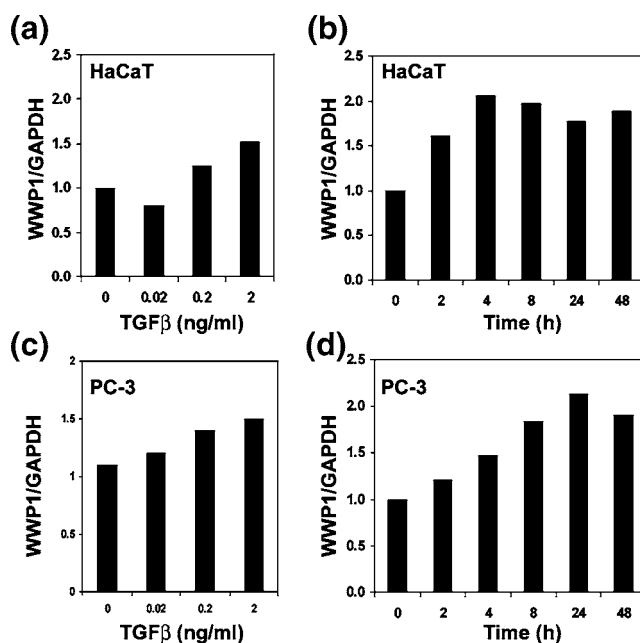


Fig. 4 TGF β induces the expression of *WWP1* mRNA in HaCaT and PC-3 cells. Expression of *WWP1* mRNA was detected by SYBR real time RT-PCR. GAPDH was used as a control to normalize the cDNA input. The primers have been described in [18, 19]. Cells were serum starved overnight before adding TGF β . (a, c) Different amounts of TGF β were used to treat HaCaT or PC-3 cells for 24 h. (b, d) Two nanograms per milliliter TGF β were used to treat HaCaT or PC-3 cells for different durations

8 Concluding remarks

After more than a decade of investigation, we are certain that Nedd4-like E3 are critical regulators of multiple cancer-related growth factor receptors and transcription factors. Several Nedd4-like E3s, such as Smurf2, WWP1, Nedd4, and Smurf1 are emerging as oncogenic factors due to their frequent deregulation in human cancer. Whether Nedd4-like E3s can be used as diagnostic markers or drug targets remains to be determined because the exact role and mechanisms of action of these E3s in different cancer types remains unclear.

Smurf2 levels have been shown to correlate with poor prognosis in patients with esophageal squamous cell carcinomas [16]. WWP1 also has the potential to be a biomarker in cancer. Gene amplification and overexpression have been detected in around half of those prostate and breast cancer samples studied [18, 19]. In breast cancer, the overexpression of *WWP1* mRNA appears preferentially in ER positive and noninvasive tumors. However, immunohistochemical staining of WWP1 in a large number of tumor samples is definitely required to confirm these promising results.

siRNA inhibition of WWP1 was shown to induce growth arrest and apoptosis [19], suggesting that WWP1 could be a potential molecular target for an anti-cancer drug. However, WWP1 showed ligase-independent proliferative activity *in vitro*, implying that an inhibitor which targets ligase activity may not be effective. In contrast, targeting protein–protein interaction between WW domains and PY motifs using non-peptide small molecular inhibitors might be a better strategy, although assuring the specificity of such constructs will be a challenge. Additionally, characterization of the mechanism by which WWP1 promotes cell proliferation and survival will aid in more rational drug design for cancer therapy. Because of their documented roles in human cancers, Nedd4 and Smurf1 also warrant further investigation as potential drug targets using similar strategies.

Nedd4-like E3s may play context-dependent roles in cancer development for a number of reasons. First, Nedd4-like E3s are ubiquitously expressed in multiple tissues. The regulation of the expression of Nedd4-like E3s under physiological and pathological conditions in different tissues remains poorly understood. Second, each Nedd4-like E3 may have multiple substrates, which, in turn, mediate different functions. Previous work indicates that different WW domains in Nedd4-like E3s display different substrate preferences *in vitro*. It is plausible that each of the Nedd4-like E3 regulate a number of different target proteins. Even though there is functional redundancy among some of the members of the Nedd4-like family of E3's, there are almost as many examples of how each of these proteins specifically acts upon certain substrates. It will take some time to clearly delineate the physiological substrates of each specific Nedd4-like E3. Finally, multiple receptors may use

the same endocytic and protein trafficking machinery that are regulated by multiple Nedd4-like E3s. How these E3s specifically affect different receptors is not clear. It is widely accepted that TGF β plays a context-dependent role during tumor development, and several Nedd4-like E3s are known to antagonize TGF β signaling. Thus, it is not surprising that different, if not opposite, roles of Nedd4-like E3s in various cancer stages and types have been observed.

As further understanding leads to the development of potential anti-cancer therapeutics that target the activities of Nedd4-like E3s, it will be necessary to validate these drugs. To that end, transgenic and gene-targeted mouse models are especially powerful tools in dissecting the physiological role of Nedd4-like E3s *in vivo*. In this regard, a high throughput gene trap library (tigm.org) will accelerate *in vivo* research efforts. All nine Nedd4-like E3s knock out ES cells are available from this library. We expect more transgenic, knockout, and knockin mouse models will be generated for characterizing the function of this interesting family in the development of cancer. In conclusion, although still in its infancy, the study of Nedd4-like E3s has the potential to provide much insight into the development of cancer diagnostics and therapeutics, which could have enormous impact on human health.

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ORIGINAL ARTICLE

The WW domain containing E3 ubiquitin protein ligase 1 upregulates ErbB2 and EGFR through RING finger protein 11

C Chen¹, Z Zhou¹, R Liu¹, Y Li¹, PB Azmi² and AK Seth²

¹The Center for Cell Biology and Cancer Research, MS355, Albany Medical College, Albany, NY, USA and ²Division of Molecular and Cellular Biology, Sunnybrook Research Institute, and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

The WW domain containing E3 ubiquitin protein ligase 1 (WWP1) is a homologous to the E6-associated protein C terminus-type E3 ligase frequently overexpressed in human prostate and breast cancers due to gene amplification. Previous studies suggest that WWP1 promotes cell proliferation and survival; however, the mechanism of WWP1 action is still poorly understood. Here, we showed that WWP1 upregulates and maintains erythroblastic leukemia viral oncogene homolog 2 (ErbB2) and epithelial growth factor receptor (EGFR) in multiple cell lines. WWP1 depletion dramatically attenuates the EGF-induced ERK phosphorylation. WWP1 forms a protein complex with RING finger protein 11 (RNF11), a negative regulator of ErbB2 and EGFR. The protein–protein interaction is through the first and third WW domains of WWP1 and the PY motif of RNF11. Although WWP1 is able to ubiquitinate RNF11 *in vitro* and *in vivo*, WWP1 neither targets RNF11 for degradation nor changes RNF11's cellular localization. Importantly, inhibition of RNF11 can rescue WWP1 siRNA-induced ErbB2 and EGFR downregulation and growth arrest. Finally, we demonstrated that RNF11 is overexpressed in a panel of prostate and breast cancer cell lines with WWP1 expression. These findings suggest that WWP1 may promote cell proliferation and survival partially through suppressing RNF11-mediated ErbB2 and EGFR downregulation.

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Keywords: WWP1; ErbB2; EGFR; RNF11; PY motif; WW domain

Introduction

The WW domain containing E3 ubiquitin protein ligase 1 (WWP1/AIP5/Tiul1) belongs to the Nedd4-like homologous to the E6-associated protein C terminus

(HECT)-type E3 family (Chen and Matesic, 2007). Nedd4-like E3s can be identified by their distinctive domain structure: an HECT domain at the C terminus for the ubiquitin transfer (Verdecia *et al.*, 2003), a C2 domain at the N terminus for calcium-dependent phospholipid binding, and 2–4 WW domains in the middle for protein–protein interaction with PY motifs (Mosser *et al.*, 1998). It has been well established that the WW domains of WWP1 can directly bind to the PY motifs of their substrates, such as Smad2 (Seo *et al.*, 2004), Smad7 (Komuro *et al.*, 2004), Runx2 (Jones *et al.*, 2006; Shen *et al.*, 2006) and KLF5 (Chen *et al.*, 2005).

To date, WWP1 has been reported to target transforming growth factor- β (TGF- β) receptor 1 (T β R1) (Komuro *et al.*, 2004), Smad7 (Komuro *et al.*, 2004), Smad2 (Seo *et al.*, 2004), Smad4 (Moren *et al.*, 2005), Runx2 (Jones *et al.*, 2006; Shen *et al.*, 2006), KLF2 (Zhang *et al.*, 2004) and KLF5 (Chen *et al.*, 2005) for ubiquitin-mediated proteolysis. Recently, WWP1 was reported to promote p53 ubiquitination and export p53 from the nucleus (Laine and Ronai, 2007). Through ubiquitinating its substrates, WWP1 negatively regulates TGF- β signaling. In line with this, we found that WWP1 is a potential oncogene that undergoes genomic amplification and overexpression in a subset of human prostate and breast cancers (Chen *et al.*, 2007a, b).

Besides T β R1, the Nedd4-like E3s have been shown to regulate the degradation of multiple membrane receptors such as the Notch receptors (Qiu *et al.*, 2000), insulin-like growth factor 1 receptor (IGF-1R) (Vecchione *et al.*, 2003), vascular endothelial growth factor receptor 2 (VEGF-R2) (Murdaca *et al.*, 2004), chemokine (C-X-C motif) receptor 4 (CXCR4) (Marchese *et al.*, 2003) and epithelial growth factor receptor (EGFR) (Courbard *et al.*, 2002; Katz *et al.*, 2002; Magnifico *et al.*, 2003). It is well established that the abnormal activation of the EGF receptors is a common theme in epithelial cancer (Yarden, 2001). Nedd4 and Itch have been demonstrated to inhibit EGFR degradation through ubiquitinating multiple adaptor proteins essential for endocytosis such as Cbls (Courbard *et al.*, 2002; Magnifico *et al.*, 2003), EPS15 (Woelk *et al.*, 2006), Hgs (Katz *et al.*, 2002) or Endophilin A1 (Angers *et al.*, 2004). However, whether WWP1 also regulates EGFRs has never been explored.

Correspondence: Dr C Chen, The Center for Cell Biology and Cancer Research, Albany Medical College, MS355, Mail Code 165, 47 New Scotland Avenue, Albany, NY 12208, USA.

E-mail: chenc@mail.amc.edu

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Really interesting new gene (RING) finger protein 11 (RNF11) is a small RING finger-type E3 ligase whose expression was deregulated in breast tumors (Kitching *et al.*, 2003; Azmi and Seth, 2005). RNF11 was proposed to enhance EGFR degradation (Azmi and Seth, 2005; Burger *et al.*, 2006). RNF11 has a PY motif and has been shown to be an interacting protein of the HECT-type E3s Smurf2 and Itch (Kitching *et al.*, 2003; Subramaniam *et al.*, 2003). By yeast two-hybrid, RNF11 was also shown to interact with WWP1 (Azmi and Seth, 2005). However, complete analysis of the relationship between WWP1 and RNF11 has been lacking, although both proteins were implicated in breast and prostate cancers. In this study, we provided multiple lines of evidence to support that WWP1 interacts with the RNF11 protein and inhibits RNF11-

mediated erythroblastic leukemia viral oncogene homolog 2 (ErbB2) and EGFR downregulation.

Results

WWP1 overexpression upregulates ErbB2 and EGFR

The *WWP1* gene is frequently amplified and overexpressed in human prostate and breast cancers (Chen *et al.*, 2007a, b). Interestingly, we found that WWP1 overexpression by lentiviruses promotes MCF10A cell proliferation in an E3 ligase activity independent manner (Chen *et al.*, 2007b). We examined the ErbB2 and EGFR protein levels in the WWP1 overexpressed MCF10A cell populations and found that both ErbB2

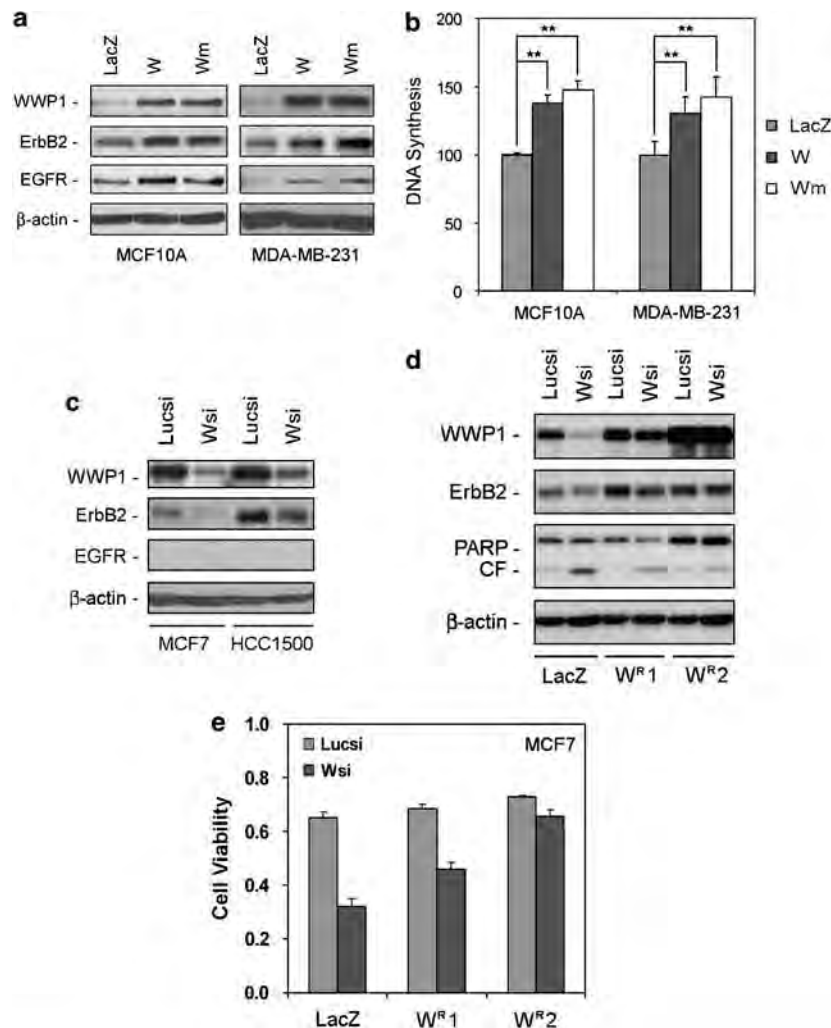


Figure 1 WW domain containing E3 ubiquitin protein ligase 1 (WWP1) regulates ErbB2/EGFR and cell proliferation/survival in breast cell lines. **(a)** WWP1 overexpression in MCF10A and MDA-MB-231 increases the protein levels of ErbB2 and EGFR in a ligase activity independent manner, as determined by western blot. **(b)** WWP1 significantly promotes DNA synthesis in MCF10A and MDA-MB-231, as determined by ³H thymidine incorporation. The LacZ control was defined to be 100. ***P* < 0.01 (*t*-test). W is the WT human WWP1, and Wm is the catalytic inactive human WWP1C890A. The cell populations with a low passage number (<3) were used in this study. **(c)** The expression levels of ErbB2 are decreased in MCF7 and HCC1500 when WWP1 is knocked down (EGFR is undetectable in these two cell lines). **(d)** Expression of siRNA resistant WWP1^R rescues WWP1 siRNA induced ErbB2 decrease and PARP cleavage in MCF7. WWP1^R and LacZ were stably introduced into MCF7 by lentiviruses. Two stable clones with different expression levels of WWP1^R were used. The overexpression of WWP1^R elevated the ErbB2 level. **(e)** WWP1^R rescues WWP1 siRNA induced loss of cell viability, as determined by the sulforhodamine-B (SRB) assay.

and EGFR are upregulated by WWP1 in a ligase activity independent manner (Figure 1a). Consistently, cell proliferation is significantly increased by WWP1, as determined by DNA synthesis (Figure 1b). Similar results were obtained in MDA-MB-231 (Figures 1a and b). To determine whether WWP1 promotes cell proliferation partially through ErbB2 and EGFR, we treated the MCF10A-LacZ and MCF10A-WWP1 cells with ErbB2 and EGFR dual kinase inhibitor Lapatinib (5–10 μ M) (Wang *et al.*, 2006) and examined EGF-stimulated DNA synthesis. As shown in Supplementary Figure S1, Lapatinib effectively blocks EGF-induced ERK phosphorylation and WWP1-induced but not overall cell proliferation in MCF10A.

WWP1 depletion downregulates ErbB2

We have previously shown that WWP1 siRNAs induce cell growth arrest and apoptosis in MCF7 and HCC1500 (Chen *et al.*, 2007b). To test whether WWP1 ablation downregulates ErbB2 and EGFR in these two cell lines, we examined the expression of ErbB2 and EGFR after WWP1 knockdown. The levels of ErbB2 protein are indeed downregulated by WWP1 siRNA in MCF7 and HCC1500, although EGFR is undetectable (Figure 1c). To further test if the ErbB2 decrease and apoptosis are really caused by WWP1 depletion rather than the off-target effect of siRNA, we stably expressed the WWP1 siRNA resistant WWP1^R and LacZ in MCF7 cells, respectively. As shown in Figures 1d and e, the WWP1 siRNA induces ErbB2 decrease, PARP cleavage, and loss of cell viability in LacZ overexpressed MCF7 cells compared with the Lucsi control. Importantly, the WWP1^R overexpression partially, even almost completely, rescues the WWP1 siRNA induced ErbB2 decrease, PARP cleavage, and apoptosis. Interestingly, WWP1^R overexpression upregulates ErbB2 in MCF7 compared with the LacZ control (Figure 1d). We noticed that the ErbB2 levels are not correlated with the WWP1 levels in two WWP1^R overexpressing clones although the WWP1 siRNA induced ErbB2 decrease and apoptosis are more completely blocked in the W^{R2} clone. These results imply that WWP1 may also promote MCF7 survival through other mechanisms besides ErbB2. Taken together, WWP1 could maintain high levels of ErbB2 and promote cell survival in cancer cells.

WWP1 depletion decreases the cell surface ErbB2/EGFR levels and EGF signaling

Both ErbB2 and EGFR are membrane receptors. To further test if the cell surface levels of ErbB2 and EGFR are regulated by WWP1, we stained the WWP1 siRNA and Lucsi-transfected MCF10A cells with PE fluorescence dye conjugated anti-ErbB2 and anti-EGFR Abs. As shown in Figure 2a, the cell surface staining for ErbB2 and EGFR is significantly decreased by WWP1 knockdown in MCF10A ($P < 0.01$). The decrease of EGFR is more obvious than the decrease of ErbB2. We further performed qRT-PCR and found that the mRNA levels of ErbB2 and EGFR are

not changed by WWP1 siRNA in MCF10A (Figure 2b). These findings suggest that the regulation of ErbB2 and EGFR by WWP1 may occur at the posttranslational level.

On ligand binding, ErbB2 and EGFR will form homo- or hetero-dimers and be autophosphorylated, which results in signaling transduction. To test whether WWP1 depletion inhibits EGF signaling, we transfected MCF10A cells with the Luc siRNA and WWP1 siRNA respectively and treated cells with 50 ng/ml EGF for different times. As reported, EGF induces rapid degradation of EGFR but not ErbB2. Consistent with previous results, WWP1 depletion decreases the levels of ErbB2 and EGFR (Figure 2c). Importantly, the activation of ERK in response to EGF is dramatically inhibited by WWP1 siRNA in terms of strength and time (Figure 2c).

WWP1 interacts with RNF11 in vivo and in vitro via WW/PY motifs

The molecular mechanism of WWP1 upregulating ErbB2 and EGFR is unknown. A potential WWP1 interacting protein RNF11 has been reported to promote EGFR degradation (Burger *et al.*, 2006). RNF11 is a Cbl-like RING finger E3 ubiquitin ligase with a PY (PPXY) motif which could interact with the WW domains of WWP1. To test whether RNF11 interacts with WWP1 through the PY/WW motifs, we performed glutathione *S*-transferase (GST) pull-down and co-immunoprecipitation (IP) assays in HEK293T cells. As shown in Figure 3a, a catalytic inactive form of mouse WWP1 (Myc-WWP1C886S) is specifically pulled down by GST-RNF11 but not GST or GST-RNF11Y40A (The PY motif is disrupted) (Subramaniam *et al.*, 2003). At the same time, GST-RNF11 but not GST-RNF11Y40A can be co-immunoprecipitated with the Myc-WWP1C886S protein in the reciprocal experiments (Figure 3b). In addition, we found that RNF11-V5 prefers to bind to the first and third WW domains of WWP1 by GST pull-down assay (Figure 3c). Furthermore, the interaction between WWP1 and RNF11 is not affected by EGF signaling in 22Rv1 cells (Supplementary Figure S2). These findings suggest that the WWP1 protein could interact with the RNF11 protein in cultured mammalian cells via the WW/PY motifs.

To determine if WWP1 directly interacts with RNF11 *in vitro*, we performed GST pull-down experiments using the purified recombinant GST-WWP1 fusion protein and the *in vitro* translated ³⁵S-labeled RNF11 protein. Both GST-WWP1 and mutant GST-WWP1C886S can pull down the RNF11 protein, but the GST protein cannot bind to the RNF11 protein under the same condition (Figure 3d). We found that RNF11Y40A is not associated with either GST-WWP1 or GST-WWP1C886S (data not shown). To test whether endogenous RNF11 interacts with endogenous WWP1, we immunoprecipitated endogenous RNF11 from MCF7 and found that the endogenous WWP1 protein is in the same complex (Figure 3e).

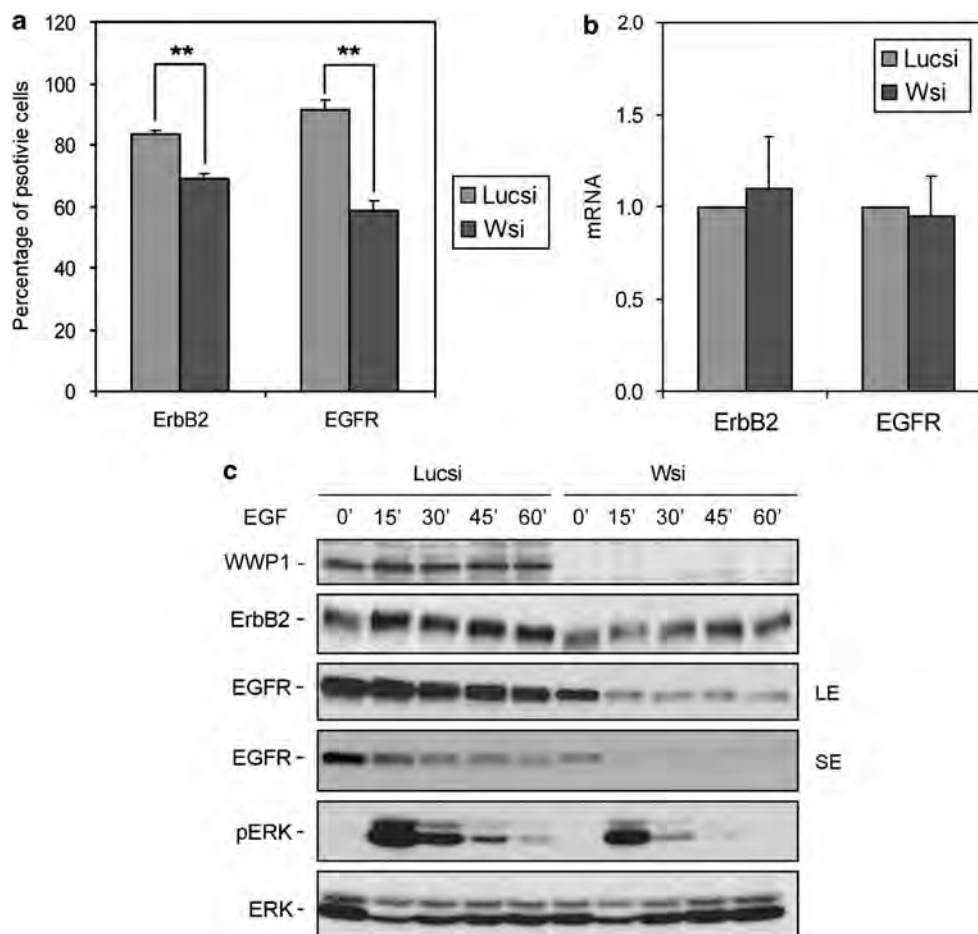


Figure 2 WW domain containing E3 ubiquitin protein ligase 1 (WWP1) depletion decreases cell surface ErbB2/EGFR levels and EGF signaling. **(a)** WWP1si decreases the cell surface ErbB2 and EGFR levels in MCF10A. Lucsi serves as the negative control siRNA. PE-conjugated IgG serves as the negative control. The fluorescence intensity was detected by flow cytometry. The percentages of ErbB2 and EGFR positive cells are significantly ($P < 0.01$, t -test) decreased by WWP1 siRNA. **(b)** The mRNA levels of ErbB2 and EGFR are not changed by WWP1 siRNA, as determined by qRT-PCR. The mRNA levels of ErbB2 and EGFR in Lucsi-transfected MCF10A cells are defined as 1. **(c)** WWP1si decreases the ErbB2 and EGFR levels and the EGF-induced ERK phosphorylation in MCF10A. A total of 50 ng/ml EGF was used to treat siRNA-transfected (for 48 h) and serum-starved (for overnight) MCF10A cells. The total ERK level serves as the loading control; LE, long exposure; SE, short exposure.

Finally, we tested if WWP1 co-localizes with RNF11 in cells. It has been established that WWP1 localizes in the membrane, endosome, or nucleus (Martin-Serrano *et al.*, 2005; Flaszka *et al.*, 2006) whereas RNF11 is located in the endosome (Anderson *et al.*, 2007) in HEK293T cells. To directly visualize WWP1 under the fluorescence microscopy, we generated a construct (Myc-mCherry-WWP1) which expresses mCherry fused WWP1. The fusion of mCherry to WWP1 does not affect the protein interaction with RNF11-V5, the function of WWP1, and subcellular localization of WWP1 (Supplementary Figure S3). We cotransfected expression plasmids for Myc-mCherry-WWP1 and RNF11-V5 into HEK293T cells and examined the subcellular localization of WWP1 and RNF11. We observed that Myc-mCherry-WWP1 is predominantly overlapping with RNF11-V5 in the endosome (Figure 3f). These findings further suggest that WWP1 may interact with RNF11 in cultured mammalian cells.

WWP1 ubiquitinates RNF11 in vitro and in vivo
To test whether WWP1 promotes RNF11 ubiquitination, we first performed an ubiquitin conjugation assay *in vitro* using the recombinant GST-WWP1 protein and *in vitro*-translated ^{35}S -labeled RNF11 and RNF11Y40A. In the presence of ubiquitination reagents from an *in vitro* ubiquitin conjugation kit, RNF11 is not self-ubiquitinated, although RNF11 is an E3 ligase. As expected, the recombinant GST-WWP1 protein significantly decreases the native wild-type (WT) RNF11 protein level and increases its ubiquitination (lane 4 in Figure 4a). A band corresponding to monoubiquitinated RNF11 was clearly detected. Under the same conditions, mutant GST-WWP1C886S (lane 5) or GST (lane 3) has no effect on RNF11 ubiquitination *in vitro*. Similarly, GST-WWP1 has no effect on the mutant RNF11Y40A protein (lane 9 in Figure 4a). These results suggest that both the ligase activity of WWP1 and protein interaction between WWP1 and RNF11 are

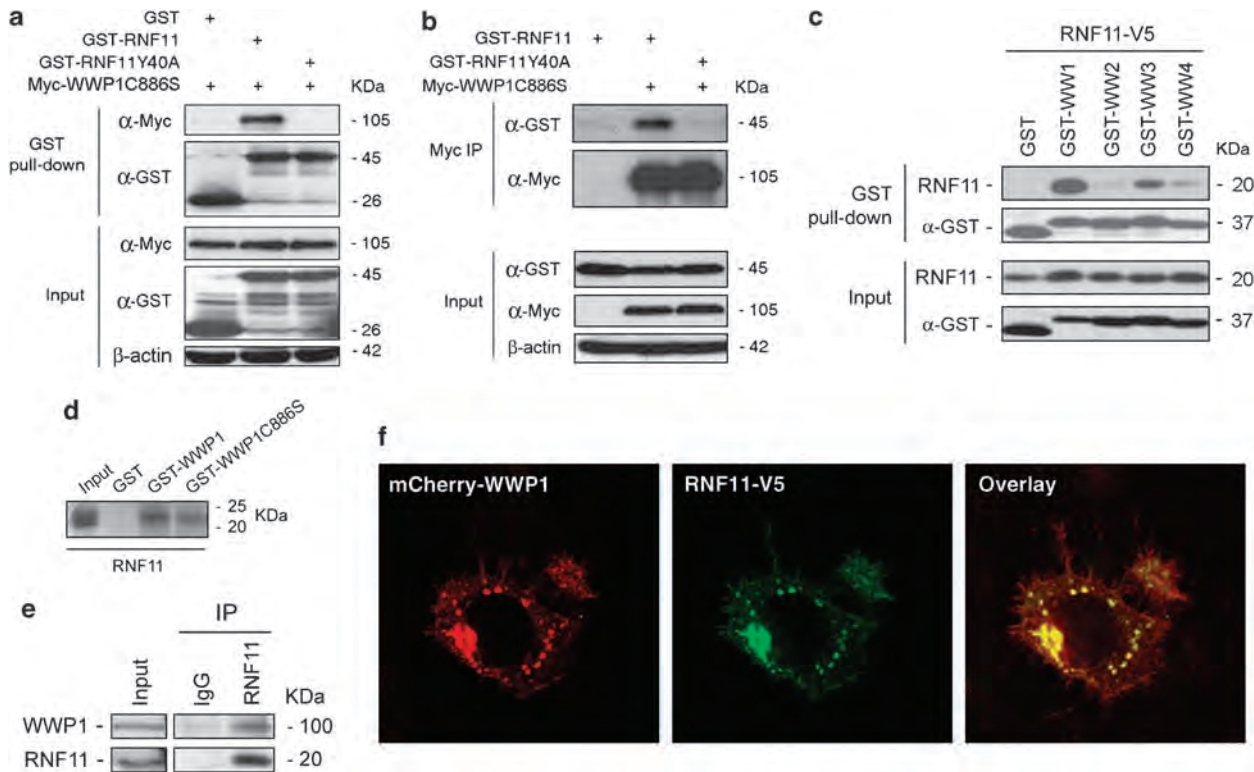


Figure 3 WW domain containing E3 ubiquitin protein ligase 1 (WWP1) interacts with RNF11 via the WW/PY motifs. **(a)** The PY motif of RNF11 is required for protein interaction with WWP1, as determined by the GST pull-down assay. Myc-WWP1C886S and GST-RNF11/GST-RNF11Y40A/GST were transfected into HEK293T for 2 days. GST fusion proteins were pulled down with Glutathione Sepharose 4B slurry beads. **(b)** The PY motif of RNF11 is required for protein interaction with WWP1, as determined by the co-IP experiment. Myc-WWP1C886S was immunoprecipitated with anti-Myc Ab and protein A beads. **(c)** RNF11 binds to the first and third WW domains of WWP1. The GST pull-down assay was performed. RNF11-V5 was probed with anti-V5 Ab. **(d)** The recombinant GST-WWP1 and GST-WWP1C886S fusion proteins pulled down the *in vitro* translated ³⁵S-labeled RNF11 protein but the GST protein did not. **(e)** Endogenous RNF11 interacts with WWP1 in MCF7. The MCF7 cell lysate from one 100 mm dish was immunoprecipitated with either 5 μl mouse anti-RNF11 antibody (Abnova) or mouse IgG. The blot was probed with rabbit anti-WWP1 and anti-RNF11 Abs. Five percent of the input cell lysate was used as the control. **(f)** Co-localization of WWP1 and RNF11 in HEK293T cells. RNF11-V5 was detected by immunofluorescence staining using anti-V5 Ab. Myc-mCherry-WWP1 can be directly visualized under the fluorescent microscope. RNF11-V5 was reported to localize to the endosome in HEK293T cells (Anderson *et al.*, 2007).

required for RNF11 to be ubiquitinated by WWP1 in this cell-free system.

To further test if WWP1 ubiquitinates the RNF11 protein *in vivo*, we transfected the expression construct for GST-RNF11 into HEK293T cells. We found that the RNF11 protein is heavily ubiquitinated without WWP1 *in vivo* (data not shown). This could be caused by RNF11 self-ubiquitination in cells because RNF11 itself is a RING finger-type E3. As mentioned above, RNF11 is not self-ubiquitinated *in vitro*. It is possible that the RNF11 matched ubiquitin conjugation enzyme E2 does not exist in the rabbit ubiquitin conjugation system. To avoid RNF11 self-ubiquitination *in vivo*, we generated a mutant RNF11 (GST-RNF11ΔR) in which the RING finger domain (94–154 residue) is deleted. Indeed, the ubiquitination of GST-RNF11ΔR is barely detected in the absence of WWP1 (Figure 4b, lane 1). Expression of WWP1 significantly increases a smear of the RNF11 protein with higher molecular weights than WT GST-RNF11ΔR (lane 2), as determined by the anti-GST Ab. Notably, the ubiquitination is not polyubiquitination but likely monoubiquitination (mUb) or

multi-mUb. Expression of mutant Myc-WWP1C886S does not induce ubiquitination of GST-RNF11ΔR (lane 3). In contrast, the original existing monoubiquitinated band disappears in the presence of WWP1C886S. These results indicate that WWP1 may induce mUb of RNF11 *in vivo*.

As RNF11 is an E3 ligase, we also tested whether RNF11 ubiquitinates WWP1 *in vivo*. The FLAG-WWP1C890A mutant was used in this experiment because WT WWP1 is self-ubiquitinated in HEK293T cells (Supplementary Figure S4). We observed that RNF11-V5 increases but RNF11ΔR-V5 (a dominant negative RNF11 mutant without the E3 ligase activity but with WWP1 interaction ability) decreases the ubiquitination of FLAG-WWP1C890A. These data suggest that RNF11 may also ubiquitinate WWP1.

WWP1 does not target RNF11 for degradation

As WWP1 is an E3 ligase for RNF11, we asked whether WWP1 targets RNF11 for ubiquitin-mediated degradation

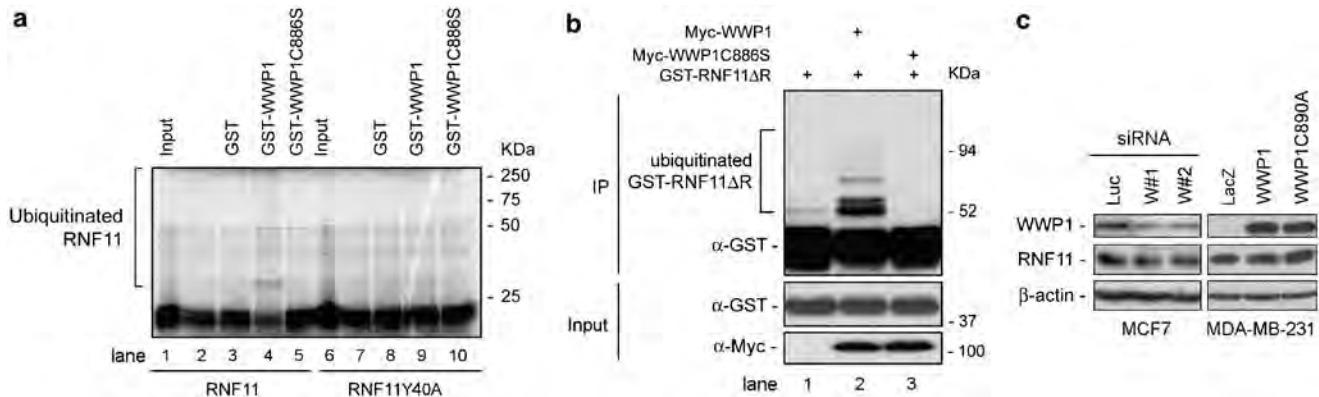


Figure 4 WW domain containing E3 ubiquitin protein ligase 1 (WWP1) ubiquitinates RNF11 but does not regulate the RNF11 protein stability. **(a)** The recombinant GST protein or GST-fused WWP1/WWP1C886S proteins were incubated with *in vitro* translated RNF11 or RNF11Y40A proteins labeled with ^{35}S , and the reaction products were subjected to polyacrylamide gel electrophoresis (PAGE) and autoradiography. 'Input' is *in vitro* translated RNF11 (lane 1) or RNF11Y40A (lane 6) proteins, which serve as negative controls. Lanes 2 and 7 also serve as negative controls because only the ubiquitin conjugation reagents but not any GST recombinant proteins were added into the reactions. **(b)** Wild-type (WT) but not catalytic inactive WWP1C886S ubiquitinates GST-RNF11ΔR in HEK293T cells. The GST-RNF11ΔR proteins were pulled down by Glutathione Sepharose 4B beads and detected by anti-GST Ab. **(c)** WWP1 does not affect the stable levels of endogenous RNF11 in breast cancer cells. Two WWP1 siRNAs were transfected into MCF7. The target sequence of W 1 siRNA is provided in experimental procedures. The target sequence of W 2 siRNA has been shown in a previous study (Chen *et al.*, 2007a). Human WWP1 and WWP1C890A overexpressing MDA-MB-231 cells have been described in Figure 1.

by the 26S proteasome. To this end, we examined whether the endogenous RNF11 level is increased when WWP1 is knocked down. We found that the RNF11 protein levels are not elevated by WWP1 siRNAs in MCF7 (Figure 4c), MCF10A (Figure 6a) or PC-3 (Supplementary Figure S5). At the same time, WWP1 overexpression does not decrease the endogenous RNF11 protein levels in MDA-MB-231 (Figure 4c). These findings suggest that WWP1 does not regulate the RNF11 stability. Similarly, RNF11 ablation does not affect the protein levels of WWP1 in MCF10A and PC-3 (Figures 5a, 6a and Supplementary Figure S5).

RNF11 downregulates ErbB2 and EGFR

To test whether RNF11 downregulates ErbB2 and EGFR, we knocked down endogenous RNF11 by two different siRNAs in MCF10A. As shown in Figure 5a, both anti-RNF11 siRNAs significantly silence the RNF11 protein expression in MCF10A. As a result, RNF11 depletion significantly elevates the ErbB2 and EGFR levels compared with the negative control Luc siRNA, as determined by western blot. Similar results were obtained from MCF7, BT20 and HeLa cancer cell lines (Supplementary Figure S6). We confirmed the upregulation of the cell surface ErbB2 and EGFR levels in MCF10A by flow cytometry (Figure 5b and Supplementary Figure S7). Similar to WWP1 overexpression, RNF11 siRNA 1 does not change the mRNA levels of ErbB2 or EGFR in MCF10A (Figure 5c) and HeLa (data not shown). When RNF11 is knocked down in MCF10A, the EGF-induced pERK levels are upregulated in terms of strength and time (Figure 5d). We noticed that RNF11 siRNA elevates the

overall ErbB2 and EGFR levels but does not block EGF-induced EGFR degradation. As expected, RNF11 ablation by siRNA promotes cell proliferation as determined by DNA synthesis (Figure 5e). Similar results were also observed in BT20 and HeLa (data not shown).

WWP1 inhibits RNF11's function

RNF11 promotes but WWP1 suppresses TGF- β signaling (Subramaniam *et al.*, 2003; Komuro *et al.*, 2004; Li and Seth, 2004; Seo *et al.*, 2004; Moren *et al.*, 2005). These reports suggest that WWP1 may inhibit RNF11's function. Interestingly, RNF11 was also reported to inhibit EGF signaling by promoting EGFR endocytosis and degradation (Azmi and Seth, 2005; Burger *et al.*, 2006). Indeed, we found that RNF11 siRNA increases both ErbB2 and EGFR and promotes cell proliferation in MCF10A (Figure 5). In contrast, WWP1 siRNA reduces both ErbB2 and EGFR (Figure 2). Thus, RNF11si should be able to rescue the WWP1si induced growth arrest and ErbB2/EGFR decrease in MCF10A, if WWP1 functions through inhibiting RNF11. To this end, we transfected RNF11si and WWP1si individually and together into the MCF10A and examined ErbB2 and EGFR levels and cell proliferation by DNA synthesis. As expected, RNF11 knockdown effectively rescues the WWP1 knockdown-induced ErbB2 and EGFR decrease (Figure 6a) and growth arrest (Figure 6b). RNF11 knockdown also effectively rescues the WWP1 knockdown-induced ErbB2 and EGFR decrease in PC-3 (Supplementary Figure S5). These results indicate that WWP1 upregulates ErbB2/EGFR and promotes cell proliferation through inhibiting RNF11.

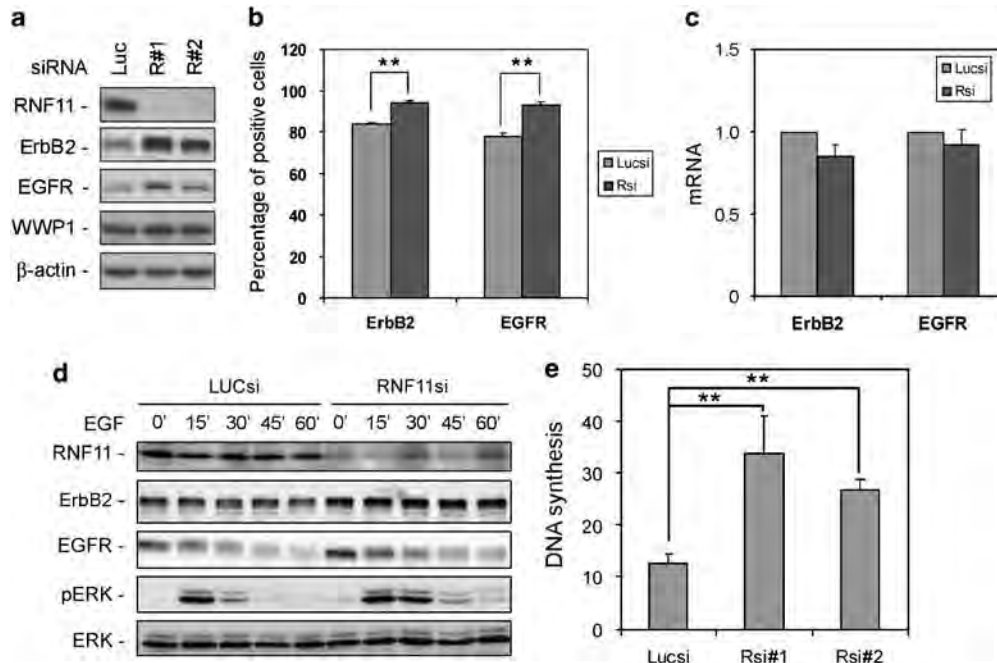


Figure 5 RNF11 knockdown increases the ErbB2 and EGFR levels and promotes cell proliferation. (a) Two RNF11 siRNAs effectively silence RNF11 expression and increase the ErbB2 and EGFR levels in MCF10A, as measured by western blot. RNF11 knockdown does not affect the WWP1 levels. (b) RNF11si 1 increased the cell surface ErbB2 and EGFR levels in MCF10A. Lucsi serves as the negative control siRNA. PE-conjugated IgG serves as the negative control. The fluorescence intensity was detected by flow cytometry. The percentages of ErbB2 and EGFR positive cells are significantly ($P < 0.01$, t -test) increased by RNF11 siRNA. (c) The mRNA levels of ErbB2 and EGFR are not changed by RNF11 siRNA 1, as determined by qRT-PCR. The mRNA levels of ErbB2 and EGFR in Lucsi-transfected MCF10A cells are defined as 1. (d) RNF11 siRNA 1 increases the ErbB2 and EGFR levels and the EGF-induced ERK phosphorylation in MCF10A. A total of 50 ng/ml EGF was used to treat siRNA-transfected (for 48 h) and serum-starved (for overnight) MCF10A cells. The total ERK level serves as the loading control. (e) Both RNF11si 1 and 2 promote DNA synthesis, as determined by ^3H -thymidine incorporation. The MCF10A cells were seeded in 24-well plates at the density of 1×10^4 per well. The cells were serum-starved at 24 h after siRNA transfection. DNA synthesis was performed for 4 h in the presence of 50 ng/ml EGF. All analyses were performed at 48 h after siRNA transfection; $**P < 0.01$ (t -test).

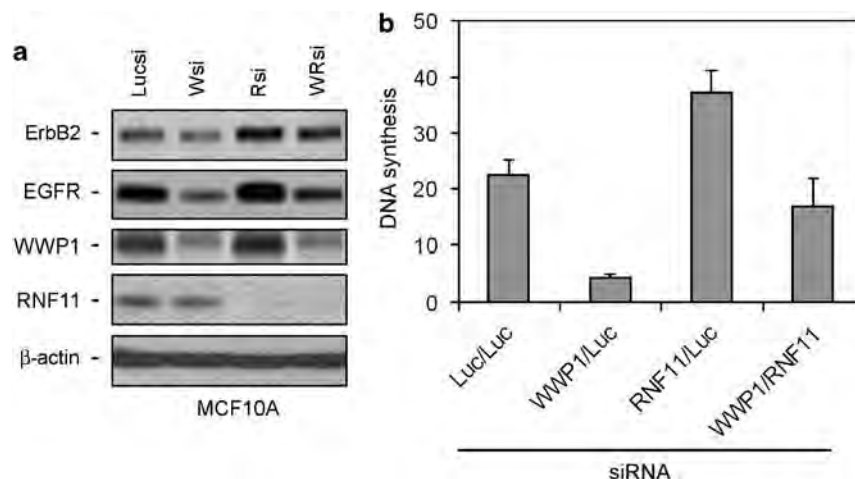


Figure 6 RNF11si rescues the WWP1si-induced ErbB2 and EGFR downregulation and growth arrest in MCF10A. (a) RNF11 siRNA 1 effectively rescues WWP1 siRNA 1 induced ErbB2 and EGFR decrease in MCF10A, as measured by western blot. (b) RNF11si 1 effectively rescues WWP1 siRNA 1 induced DNA synthesis decrease in MCF10A, as measured by ^3H -thymidine incorporation. Both WWP1 siRNA and RNF11 siRNA were transfected at 100 nM final concentration for 48 h.

RNF11 is overexpressed in prostate and breast cancer cell lines

Although RNF11 is a negative regulator of ErbB2 and EGFR, RNF11 has been reported to be overexpressed

in breast cancer as detected by immunohistochemistry (IHC) (Kitching *et al.*, 2003; Azmi and Seth, 2005). To confirm this result, we examined the RNF11 levels in breast cancer cell lines by qRT-PCR in 17 breast cancer

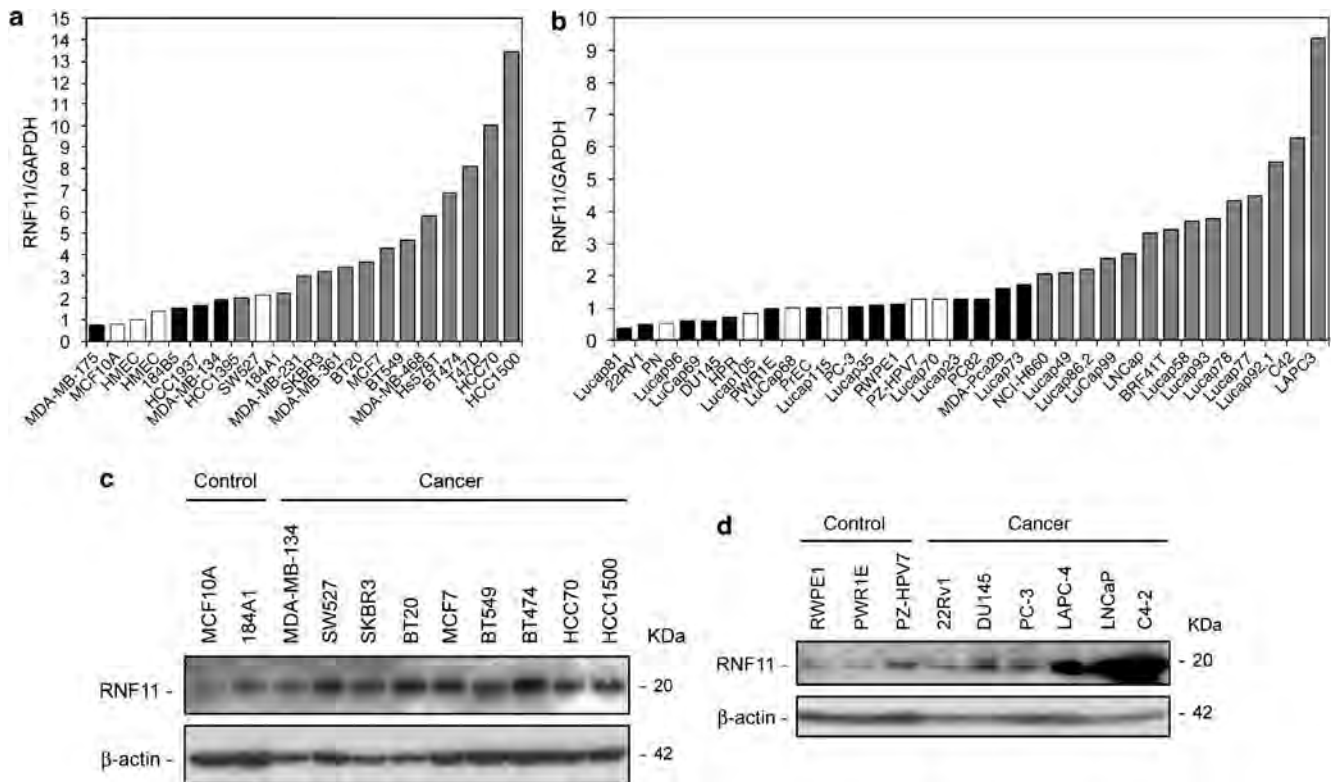


Figure 7 The expression of RNF11 in breast and prostate cancer cell lines. **(a)** RNF11 mRNA is upregulated in breast cancer cell lines, as determined by qRT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serves as the loading control. White bars represent nontransformed cell lines, solid bars represent cancer cell lines without RNF11 overexpression, and gray bars represent cancer cell lines with RNF11 overexpression. **(b)** RNF11 mRNA is upregulated in prostate cancer cell lines, as determined by qRT-PCR. **(c)** The RNF11 protein level is upregulated in breast cancer cell lines compared with two immortalized breast epithelial cell lines, as determined by western blot. β -actin serves as the loading control. **(d)** The RNF11 protein level is upregulated in prostate cancer cell lines compared with three immortalized prostate epithelial cell lines, as determined by western blot.

cell lines and four nontransformed breast epithelial cell lines. RNF11 mRNA is upregulated at least twofold in 13 breast cancer cell lines compared with the average levels of RNF11 mRNA in four nontransformed cell lines (Figure 7a). Similarly, we found that RNF11 mRNA is upregulated in 50% (14/28) of prostate cancer cell lines/xenografts (Figure 7b). RNF11 overexpression in breast and prostate cancer cell lines was further confirmed at the protein level (Figures 7c and d). Interestingly, WWP1 expression is also upregulated in these breast and prostate cancer cell lines except BT20 (Chen *et al.*, 2007a, b). In a panel of breast cancer cell lines, the expression levels of WWP1 appear to be associated with the ErbB2 levels (Supplementary Figure S8).

Discussion

The *WWP1* gene undergoes frequent genomic amplification at 8q21 and concomitant overexpression in human prostate and breast cancers. Functionally, forced overexpression of WWP1 promotes cell proliferation in an E3 ligase independent manner, and WWP1 knock-down significantly suppresses cancer cell proliferation

and induces apoptosis. Although WWP1 is a potential oncogene in prostate and breast cancers, the molecular mechanism of WWP1 action is not very clear. In this study, we found that WWP1 overexpression upregulates ErbB2 and/or EGFR in MCF10A, MDA-MB231 and MCF7 (Figures 1 and 2). In addition, WWP1 knock-down downregulates ErbB2 and/or EGFR in MCF7, HCC1500, MCF10A and PC-3, suggesting that WWP1 may promote cell proliferation and survival partially through positively regulating ErbB receptors.

We provide several lines of evidence here to support that WWP1 upregulates ErbB2 and EGFR indirectly through inhibiting RNF11. First, WWP1 interacts with RNF11 through the WW/PY motifs. Second, both WWP1 overexpression and RNF11 ablation increase the ErbB2 and EGFR levels. Most importantly, RNF11 ablation can rescue the WWP1 knockdown induced ErbB2 and EGFR downregulation. The notion that WWP1 inhibits RNF11 is further supported by the facts that RNF11 promotes (Subramaniam *et al.*, 2003; Li and Seth, 2004) but WWP1 suppresses TGF- β signaling (Komuro *et al.*, 2004; Seo *et al.*, 2004; Moren *et al.*, 2005). Finally, co-expression of WWP1 and RNF11 provides the opportunity for WWP1 to inhibit RNF11 in prostate and breast cancer cells. These findings suggest that WWP1 may suppress the RNF11

activity and then upregulate the ErbB2 and/or EGFR levels. Thus, WWP1 may function through RNF11 to promote EGF signaling and to inhibit TGF- β signaling.

The protein interaction between WWP1 and RNF11 is supported by several lines of results. First, RNF11 was reported to be a WWP1 interacting protein by two independent yeast two-hybrid experiments (Azmi and Seth, 2005; Rual *et al.*, 2005). Second, we demonstrated that WWP1 interacts with RNF11 *in vivo* by GST pull-down assays and reciprocal co-IP experiments (Figures 3a–c). The PY motif of RNF11 and the WW domains of WWP1 are responsible for the protein interaction. We further showed that the GST-WWP1 protein directly interacts with the RNF11 protein *in vitro* (Figure 3d). Importantly, we demonstrated that endogenous WWP1 interacts with endogenous RNF11 *in vivo* (Figure 3e). Finally, WWP1 and RNF11 co-localize in the endosomal system as determined by immunofluorescence staining (Figure 3f). Taken together, the WWP1 protein interacts with the RNF11 protein through the WW/PY motifs.

The consequence of protein interaction between WWP1 and RNF11 is that both proteins can be mutually ubiquitinated (Figure 4 and Supplementary Figure S4). However, degradation of the WWP1 and RNF11 proteins is not affected. WWP1 upregulates both ErbB2 and EGFR in MCF10A and MDA-MB-231 in a ligase activity independent manner (Figure 1a) suggests that the ubiquitination of RNF11 may not be essential for WWP1 to upregulate ErbB2 and EGFR. Under physiological conditions, we cannot exclude that WWP1 regulates the RNF11 activity through mUb or multi-mUb because WWP1 is rarely mutated, at least in prostate cancer cells (Chen *et al.*, 2007a). The RNF11 protein is an ubiquitin receptor containing an ubiquitin-interacting motif. Ubiquitin receptors frequently undergo mUb, which contributes to membrane receptor endocytosis and degradation (Hoeller *et al.*, 2006). It was proposed that the mUb of ubiquitin receptors blocks the endocytosis-signaling transduction because of intramolecular interactions between ubiquitins and their ubiquitin-binding domains (Hoeller *et al.*, 2006). Whether mUb of RNF11 inactivates the RNF11 activity of downregulating ErbB2 and EGFR requires further studies.

WWP1 does not change the subcellular localization of RNF11. RNF11 was shown to localize to endosomal membranes (Anderson *et al.*, 2007). WWP1 can also be recruited to the endosome (Plant *et al.*, 1997; Martin-Serrano *et al.*, 2005). WWP1 has been reported to export p53 out of the nucleus (Laine and Ronai, 2007). However, we found that WWP1 does not alter the RNF11 localization (Figure 3e and data not shown). Thus, a possible explanation is that the WWP1 binding may be sufficient to affect the RNF11 activity.

RNF11 has been proposed to downregulate EGFR through targeting AMSH (an EGFR deubiquitinating enzyme) for degradation by forming a complex with Smurf2 (Burger *et al.*, 2006). Whether the WWP1 and RNF11 complex targets AMSH for degradation needs further investigation. Several other molecules involved

in EGFR endocytosis, including EPS15 and Cbl, may also be regulated by WWP1 and RNF11. EPS15 and Cbl have been demonstrated to be RNF11-interacting proteins in yeast two-hybrid experiments (Azmi and Seth, 2005). WWP1 has been shown to increase the ubiquitination of EPS15 (Woelk *et al.*, 2006; Chen and Matesic, 2007). In addition, a WWP1 family member, Itch/AIP4, has been shown to promote Cbl ubiquitination (Magnifico *et al.*, 2003). Therefore, we cannot exclude that WWP1 also upregulates ErbB2 and EGFR through RNF11 independent mechanisms, such as EPS15 and Cbl. As RNF11 ubiquitinates WWP1, it is also possible that RNF11 inhibits the WWP1 activity. The mechanism by which RNF11 regulates ErbB2 and EGFR receptors remains to be confirmed by independent studies.

The RNF11 protein has been shown to be overexpressed in breast tumors by IHC (Subramaniam *et al.*, 2003). RNF11 may also be involved in Parkinson's disease (Anderson *et al.*, 2007). We found that WWP1 and RNF11 are concomitantly upregulated in some prostate and breast cancer cell lines (Figure 7). It is possible that RNF11 is neutralized by elevated WWP1 in prostate and breast cancer cell lines. The mechanism of RNF11 overexpression in cancer cells is not clear, although the WWP1 overexpression is caused by gene amplification. The expression correlation between WWP1 and ErbB2 still need to be validated in breast tumors by IHC.

In summary, we found that WWP1 upregulates both ErbB2 and EGFR receptors through inhibiting the RNF11 activity. Given the important role of ErbB2 and EGFR in cell proliferation, survival and tumorigenesis, our findings that WWP1 and RNF11 regulate the ErbB2 and EGFR levels may have a profound impact in understanding the role of WWP1 and RNF11 in the development and progression of human cancer or other diseases.

Materials and methods

Antibodies and reagents

The anti-GST (no. G7781), anti- β -actin (no. A5441), anti-FLAG and anti-V5 antibodies (Abs) were purchased from Sigma (St Louis, MO, USA). The anti-Myc, anti-PARP, anti-pERK and anti-ERK Abs are from Cell Signaling (Danvers, MA, USA). The anti-ErbB2 Ab, anti-HA Ab and protein A/G Plus-agarose IP reagent (sc-2003) are from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The anti-EGFR Ab, R-phycoerythrin (PE)-conjugated anti-EGFR and anti-ErbB2 Abs and IgG2b κ isotype control are from BD Biosciences (San Jose, CA, USA). The anti-WWP1 Ab is from Novus Biologicals Inc. (Littleton, CO, USA). The rabbit anti-RNF11 Ab has been described in a previous study (Subramaniam *et al.*, 2003). The mouse anti-RNF11 antibody was purchased from Abnova (Taiwan). All anti-WWP1 and anti-RNF11 siRNA were purchased from Dharmacon (Lafayette, CO, USA). The target sequence for WWP1 siRNA is 5'-GACCAAAGCTTTCCTTGAT-3'. The target sequence for RNF11 siRNA 1 is 5'-GATGACTGGTTGATGAGAT-3'. The target sequence for RNF11 siRNA 2 is 5'-TAGGATAGCTCAAAGAATA-3'. EGF was purchased from Peprotech Inc.

(Rocky Hill, NJ, USA). Lapatinib was purchased from LC laboratories (Woburn, MA, USA).

Plasmid constructions

Myc-WWP1 (mouse), Myc-WWP1C886S (mouse), WWP1 (human) and WWP1C890A (human) have been described in previous studies (Chen *et al.*, 2005, 2007b). A plasmid pRSET-BmCherry, expressing a red fluorescence protein mCherry, was kindly provided by Dr Roger Y Tsien (Shaner *et al.*, 2004). The mCherry gene was amplified with primers mCh-F and mCh-R (all primers are listed in Supplementary Table 1) and cloned into the pCMV-Myc-WWP1 vector. The resulting construct Myc-mCherry-WWP1 was confirmed by DNA sequencing. To generate GST-RNF11ΔR, the cDNA encoding the first 93 amino acids of RNF11 was amplified and cloned into the pEBG vector. RNF11-V5 and RNF11ΔR-V5 were amplified with primers RNF11-F and RNF11-V5-R or RNF11ΔR-V5-R and cloned into the pLenti6/V5 vector. The WW domains 1–4 from human WWP1 were amplified by PCR using the corresponding primers listed in Supplementary Table 1 and cloned into the pEBG vector individually.

Mutagenesis

To generate WWP1 siRNA resistant WWP1 cDNA, we silently mutated five nucleotides within the siRNA target sequence (from 5'-GCTTTCCTTGAT-3' to 5'-GCCTTTTGAC-3'), using a PCR-based approach.

Cell culture and transfection

The HEK293T and MDA-MB-231 cells were maintained in DMEM media with 5% FBS and 1% penicillin and streptomycin in the incubator with 5% CO₂. MCF10A, MCF7, HCC1500 and PC-3 cell lines were cultured as described in our previous studies (Chen *et al.*, 2007a,b). All plasmids and siRNAs were transfected into cells using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

Measurement of the cell surface ErbB2 and EGFR level by flow cytometry

MCF10A cells were transfected with different siRNAs for 2 days. The cells were trypsinized, centrifuged and resuspended in the PBS buffer with 2% BSA (80 μl for 1 million cells). The cells were incubated with 20 μl anti-ErbB2, anti-EGFR Abs or IgG for 30 min at 4 °C. Following that, the unbound Abs were removed by centrifuge. The cells were washed once and resuspended with the PBS buffer with 2% BSA for flow cytometry.

GST pull-down and co-immunoprecipitation

The GST pull-down assay and IP using the anti-Myc Ab was performed as described in our previous study (Chen *et al.*, 2005). For the GST pull-down assay *in vitro*, RNF11 and RNF11Y40A proteins were translated *in vitro* using the TNT

Quick Coupled Transcription/Translation Systems (Promega, WI, USA) in the presence of ³⁵S-methionine (MP Biomedicals, OH, USA).

Ubiquitin conjugation assay

The Ubiquitin-Protein Conjugation Kit (BostonBiochem, MA, USA) was used for the *in vitro* ubiquitination assay as described in our previous study (Chen *et al.*, 2005). For the RNF11 and WWP1 ubiquitin assay *in vivo*, HEK293T cells were transfected with HA-Ub and other constructs as necessary. At 48 h after transfection, the cells were collected and pulled down with Glutathione Sepharose 4B beads or FLAG-M2 agarose beads under denaturing conditions. Eluted proteins were subjected to immunoblotting.

qRT-PCR

Total RNA was extracted using the Trizol reagent (Invitrogen). The cDNA was prepared by using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative RT-PCR was performed using the Absolute QPCR SYBR green fluorescein mixes (ABgene, Surrey, UK). Primer sequences for RNF11 are shown in Supplementary Table 1. All qPCRs were performed in duplicate. The average ratio of RNF11 to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control in normal, primary and immortalized epithelial cells was defined as 1, and the ratios for other samples were normalized accordingly. RNF11 overexpression was defined in a sample when the ratio of RNF11 to GAPDH was ≥2.

Abbreviations

EGFR, epithelial growth factor receptor; ErbB2, erythroblastic leukemia viral oncogene homolog 2; GST, glutathione S-transferase; HECT, homologous to the E6-associated protein C terminus; IHC, immunohistochemistry; IP, immunoprecipitation; mUb, monoubiquitination; RING, really interesting new gene; RNF11, RING finger protein 11; TGF-β, transforming growth factor-β; WT, wild type; WWP1, WW domain containing E3 ubiquitin protein ligase 1.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)

WW domain-containing E3 ubiquitin protein ligase 1 targets p63 transcription factor for ubiquitin-mediated proteasomal degradation and regulates apoptosis

Y Li¹, Z Zhou¹ and C Chen^{*1}

WWP1 E3 ubiquitin ligase has previously been shown to be frequently amplified and overexpressed in prostate and breast cancers. However, the mechanism of WWP1 action is still largely unknown. p63, a member of the p53 family of transcription factors, has an important function in tumor development by regulating apoptosis. Using alternative promoters, p63 can be expressed as Δ Np63 and TAp63. Increasing evidence suggests that TAp63 sensitizes cells to apoptosis but Δ Np63 has an opposite function. In this study, we show that WWP1 binds, ubiquitinates, and destructs both Δ Np63 α and TAp63 α . The protein–protein interaction occurs between the PY motif of p63 and the WW domains of WWP1. The knockdown of WWP1 by siRNA increases the endogenous Δ Np63 α level in the MCF10A and 184B5 immortalized breast epithelial cell lines and confers resistance to doxorubicin-induced apoptosis. On the other hand, the knockdown of WWP1 increases the endogenous level of TAp63 α , induces apoptosis, and increases sensitivity to doxorubicin and cisplatin in the HCT116 colon cancer cell line in a p53-independent manner. Finally, we found that DNA damage chemotherapeutic drugs induce WWP1 mRNA and protein expression in a p53-dependent manner. These data suggest that WWP1 may have a context-dependent role in regulating cell survival through targeting different p63 proteins for degradation.

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The WW domain containing E3 ubiquitin protein ligase 1 (WWP1) belongs to the C2-WW-HECT type E3 family, which comprises eight other members including NEDD4, AIP4/Ithc, SMURF1, and SMURF2.¹ All family members share a distinctive domain structure: a C2 domain at the N terminus for calcium-dependent phospholipid binding, 2–4 WW domains in the middle for protein–protein interaction with PY motifs, and a HECT domain at the C terminus for the ubiquitin transfer. Four family members including WWP1,^{2,3} NEDD4,⁴ SMURF1,⁵ and SMURF2,⁶ have been shown to be overexpressed in different tumor types.

WWP1 is a potential oncogene that undergoes genomic amplification and overexpression in a subset of prostate and breast cancers.^{2,3} The WWP1 gene is located at 8q21, a chromosomal region frequently amplified in human prostate and breast cancers. About 31–51% of cancer samples show gene copy number gains for WWP1. Furthermore, WWP1 is overexpressed in 58–60% of prostate and breast cancer samples. Functionally, WWP1 knockdown significantly suppresses cell proliferation and/or induces apoptosis in several prostate and breast cancer cell lines,^{2,3} suggesting that WWP1 could be a promising molecular target for cancer therapy.

WWP1 is an intrinsic E3 ubiquitin ligase for multiple important proteins involved in tumorigenesis. The transform-

ing growth factor- β signaling pathway is well known to suppress epithelial proliferation and induce apoptosis but promote tumor development at later stage. Several studies suggest that WWP1 negatively regulates the TGF- β signaling by targeting its molecular components, including TGF- β receptor 1 (T β R1),⁷ Smad2,⁸ and Smad4⁹ for ubiquitin-mediated degradation. In addition, WWP1 has been reported to target several oncogenic factors such as Notch,¹⁰ Runx2¹¹ and KLF5¹² for ubiquitin-mediated proteolysis. Recently, WWP1 has been demonstrated to inhibit p53 activity through exporting p53 from the nucleus by ubiquitination.¹³ However, the role of WWP1 in tumorigenesis remains to be elucidated.

The p63 transcription factor, a member of the p53 family, shares DNA binding, oligomerization and possible transactivation (TA) domains with p53 and p73.¹⁴ Using alternative promoters, p63 can be expressed as Δ Np63 and TAp63 that have opposite functions in transcription control.¹⁵ There are three isoforms (α , β , γ) for both TAp63 and Δ Np63 because of the RNA splicing.¹⁶ All TAp63 isoforms contain an N-terminal p53-like transactivation domain which can transactivate traditional p53 target genes. Like p53, TAp63 promotes apoptosis through the death receptor and mitochondrial pathways. In contrast to TAp63, increasing evidence suggests that Δ Np63 isoforms could be oncoproteins with an anti-apoptotic activity. All Δ Np63 isoforms lack the TA domain but

¹The Center for Cell Biology and Cancer Research, MS355, Albany Medical College, Albany, NY, USA

*Corresponding author: C Chen, The Center for Cell Biology and Cancer Research, MS355, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208, USA. Fax: + 518 262 5669; E-mail: chenc@mail.amc.edu

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Abbreviations: Ab, antibody; CHX, cycloheximide; Cpt, cisplatin; DBD, DNA-binding domain; ER, estrogen receptor; HECT, homologous to the E6-associated protein carboxyl terminus; GST, glutathione S-transferase; IP, immunoprecipitation; RACK1, receptor of activated protein C kinase; SCC, squamous cell carcinoma; TA, transactivation; WT, wild type; WWP1, WW domain-containing E3 ubiquitin protein ligase 1

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still contain the DNA-binding domain and oligomerization domain, suggesting that Δ Np63 may function as dominant-negative forms of TAp63. Indeed, the Δ Np63 isoforms have been shown to act as transcriptional repressors both *in vitro* and *in vivo* and strongly oppose the function of TAp63, Tap73 and p53.¹⁶ Numerous studies have shown that Δ Np63 α is the predominate isoform expressed in epithelial tissues including epidermis, prostate and breast.¹⁶ p63 knockout mice fail to develop skin, prostate and mammary glands among other defects because of depletion of stem cells.^{17,18} Similarly, inhibition of the endogenous Δ Np63 α expression by RNAi induces epithelial apoptosis.^{19,20} In addition, the ectopic expression of Δ Np63 in fibroblast cells induces anchorage-independent growth and tumor growth in nude mice.²¹

All p53 family members are degraded through the ubiquitin–proteasome pathway.²² Multiple E3 ligases including Mdm2, ARF-BP, Cop1 and Pirh2 have been demonstrated to target p53 for ubiquitin-mediated degradation.²² The receptor of activated protein C kinase–Elongin-C/B ubiquitin ligase complex has been proposed to be an E3 ligase for Δ Np63 α .²³ Recently, two WWP1 family members, Itch and Nedd4 have been shown to promote ubiquitination and degradation of the p63 proteins.^{24,25} In addition, the inhibition of Itch potentiates the killing effect of doxorubicin in HeLa cells.²⁶

Given the frequent expression alteration of WWP1 in human cancers, it is important to know whether WWP1 regulates apoptosis through promoting p63 degradation. Here, we show that WWP1 targets both Δ Np63 α and TAp63 α

for ubiquitin-mediated proteasomal degradation. Importantly, we demonstrate that WWP1 regulates apoptosis and drug sensitivity in a p63-dependent manner. Lastly, we found WWP1 is induced by DNA damage therapeutic drugs in a p53-dependent manner. These findings help us understand the mechanism of WWP1 action in human cancer and may provide better designs for future cancer treatment.

Results

WWP1 interacts with p63 α through the WW/PY motifs. The p63 α protein has been shown to interact with Itch and Nedd4 through the PY/WW motif interaction.^{24,25} To test whether WWP1 interacts with p63 α , we first tested if p63 α can be co-immunoprecipitated with WWP1. A plasmid expressing Myc-WWP1C886S (a catalytic inactive mouse WWP1 mutant) and plasmids expressing either FLAG- Δ Np63 α or FLAG-TAp63 α were transfected into LinX cells. Myc-WWP1C886S was efficiently immunoprecipitated by the anti-Myc antibody (Figure 1a). We found that both FLAG- Δ Np63 α and FLAG-TAp63 α are co-immunoprecipitated with Myc-WWP1C886S. The anti-Myc antibody itself cannot immunoprecipitate the p63 α proteins without the expression of Myc-WWP1C886S, suggesting that the interactions are specific.

Then we tested whether the protein–protein interaction between WWP1 and p63 α is through the WW/PY motifs. We

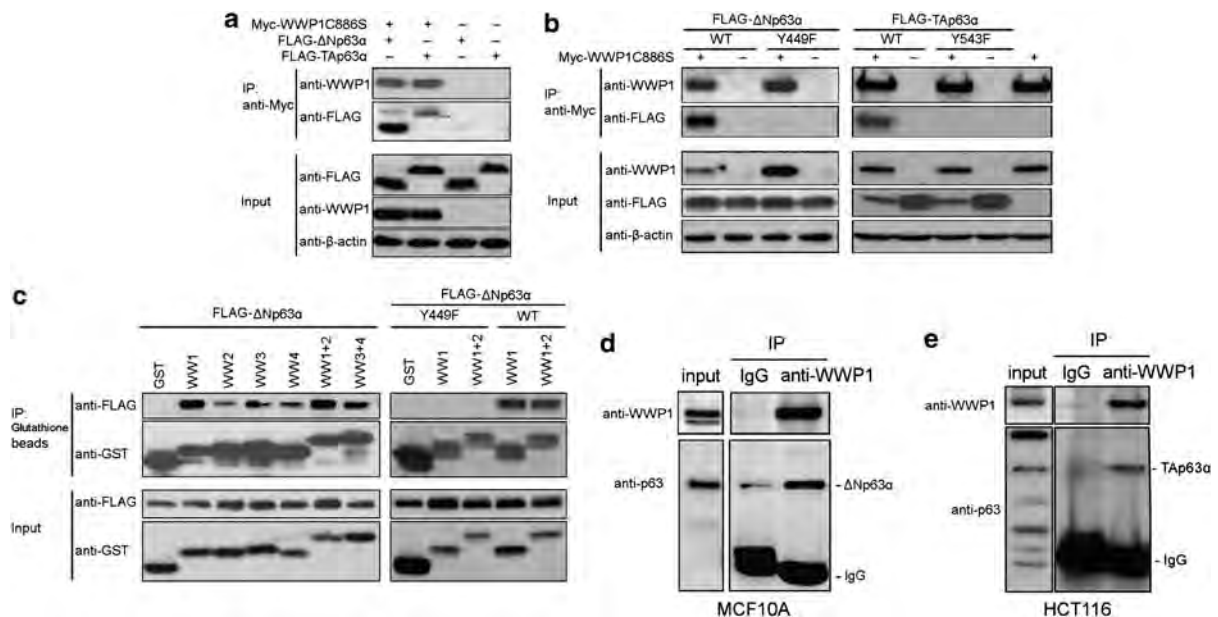


Figure 1 The WWP1 protein interacts with p63 α proteins through the WW/PY motifs in mammalian cells. **(a)** Both FLAG- Δ Np63 α and FLAG-TAp63 α are co-precipitated with Myc-WWP1C886S. LinX cells were co-transfected with different combinations of expression plasmids for Myc-WWP1C886S, FLAG- Δ Np63 α and FLAG-TAp63 α . IP was performed using anti-Myc Ab. Myc-WWP1C886S was probed by anti-WWP1 Ab. The catalytic inactive WWP1 mutant was used to avoid p63 degradation by WT WWP1. β -actin serves as a loading control for the input. **(b)** Both FLAG- Δ Np63 α and FLAG-TAp63 α interact with WWP1 through the PY motif. FLAG- Δ Np63 α Y449F and FLAG-TAp63 α Y543F are two p63 α mutants in which the PY motifs are disrupted by substituting the Tyr (Y) residue with the Phe (F) residue. The Myc-WWP1C886S-transfected LinX (without FLAG-p63 α) was used as a negative control. **(c)** WWP1 binds to FLAG- Δ Np63 α but not FLAG- Δ Np63 α Y449F through WW domains, as determined by GST pull-down assays. Four WW domains of WWP1 were individually or collectively expressed as GST fusion proteins in LinX cells. **(d)** The endogenous WWP1 protein forms a complex with the endogenous Δ Np63 α protein in MCF10A. The WWP1 protein was probed with the rabbit anti-WWP1 antibody. The same amount of mouse IgG nonspecifically immunoprecipitated few Δ Np63 α after extensive washing. However, anti-WWP1 Ab immunoprecipitated much more Δ Np63 α than the IgG control under the same conditions. **(e)** The endogenous WWP1 protein forms a complex with the endogenous TAp63 α protein in HCT116

mutated the PY motifs in p63 α (Y449F for Δ Np63 α and Y543F for TAp63 α) and performed immunoprecipitation (IP) with these mutants. We found that both PY motif-mutated p63 α proteins cannot efficiently interact with Myc-WWP1C886S (Figure 1b), suggesting that the PY motifs in Δ Np63 α and TAp63 α are required to interact with WWP1. Following that, we asked which WW domain of WWP1 participates in the protein interaction with p63 α . We fused each of the four WW domains of WWP1 to the C terminus of GST and performed GST pull-down assays with FLAG- Δ Np63 α in LinX cells. As shown in Figure 1c, GST itself does not pull down any FLAG- Δ Np63 α , but all four GST-WW proteins pull down different amounts of FLAG- Δ Np63 α . The GST-WW1 protein pulls down much more FLAG- Δ Np63 α than the rest of GST-WW proteins, although the expression levels of GST-WW and FLAG- Δ Np63 α are similar in the different groups. Consistently, GST-WW (1–2) pulls down more FLAG- Δ Np63 α than GST-WW (3–4). These findings suggest that the first WW domain may have a major function for p63 α binding. Consistent with the results in Figure 1b, both GST-WW1 and GST-WW (1–2) pull down WT but not the PY motif-mutated Δ Np63 α (Figure 1c, right panel). Taken together, the protein interaction between WWP1 and p63 α is through the WW/PY motifs.

Finally, we immunoprecipitated the endogenous WWP1 proteins from MCF10A by using anti-WWP1 Ab and found that the endogenous Δ Np63 α protein is in the same complex (Figure 1d). Similarly, we detected protein interaction between endogenous WWP1 and endogenous TAp63 α in HCT116 (Figure 1e). These results suggest that the protein interaction between WWP1 and p63 α could occur at the physiological level.

WWP1 ubiquitinates p63 α in cultured mammalian cells. As the WWP1 E3 ligase interacts with both Δ Np63 α and TAp63 α , we next determined whether WWP1

ubiquitinates the p63 α proteins in mammalian cells. To this end, we transfected the expression constructs for WT hWWP1 or the catalytic inactive mutant hWWP1C890A, FLAG- Δ Np63 α or FLAG-TAp63 α , and Myc-Ub into LinX cells. We performed IP with the anti-FLAG antibody conjugated M2 beads under a denaturing condition to eliminate any p63 α -associated proteins through non-covalent bonds. The ubiquitin-conjugated p63 α proteins were detected by western blot with anti-Myc Ab. As shown in Figure 2a, WT WWP1 significantly increases the ubiquitination of both TAp63 α and Δ Np63 α compared with the vector control, whereas the catalytic inactive WWP1C890A does not. We noticed that WWP1 shows a higher ubiquitination activity towards the Δ Np63 isoform compared with the TAp63 isoform. The format of p63 ubiquitination by WWP1 is most likely polyubiquitination because a smear of band above the unmodified p63 α was detected for both TAp63 α and Δ Np63 α . These results indicate that WWP1 polyubiquitinates both Δ Np63 α and TAp63 α through its E3 ligase activity.

In addition, we examined the ubiquitination of the PY motif-mutated Δ Np63 α Y449F and TAp63 α Y543F by WWP1. As shown in Figure 2b, WWP1 only ubiquitinates WT p63 α but not PY motif-mutated p63 α under the same conditions. We conclude that the protein interaction is essential for WWP1 to ubiquitinate p63 α .

To investigate whether endogenous WWP1 contributes to the endogenous Δ Np63 α ubiquitination, we knocked down WWP1 in MCF10A by a WWP1 siRNA and examined the ubiquitination of Δ Np63 α . Compared with the Luc siRNA, the WWP1 siRNA efficiently silenced the WWP1 protein expression, elevated the Δ Np63 α protein level and decreased the ubiquitinated Δ Np63 α .

WWP1 promotes p63 α proteasomal degradation. To test whether WWP1 targets p63 α for degradation, we first

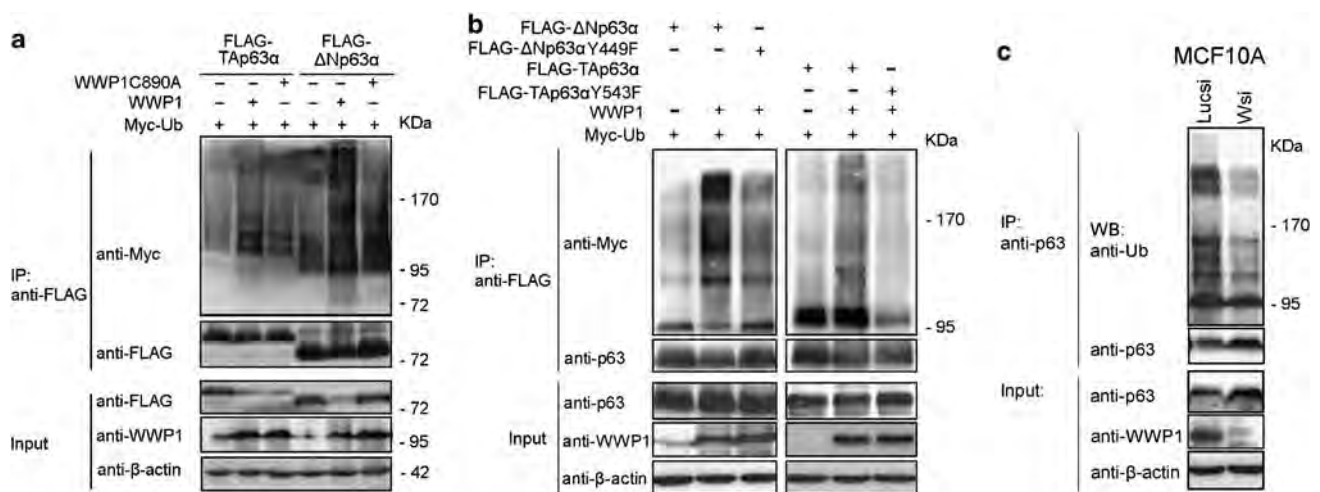


Figure 2 WWP1 ubiquitinates p63 α proteins in mammalian cells. (a) WWP1 ubiquitinates both TAp63 α and Δ Np63 α using its E3 ligase activity. LinX cells were co-transfected with expressing plasmids for Myc-Ub, WWP1, WWP1C890A, FLAG- Δ Np63 α and FLAG-TAp63 α , as indicated. The cells were treated with 20 μ M proteasome inhibitor MG132 overnight to accumulate the ubiquitinated p63 α before harvest. The IP was performed with the anti-FLAG M2 beads under denaturing conditions. Immunoblotting was performed with the indicated Abs. The ubiquitin modified p63 α proteins were detected by anti-Myc Ab. As majority of FLAG- Δ Np63 α is polyubiquitinated by WWP1, much less unmodified FLAG- Δ Np63 α was detected when WWP1 is co-transfected. (b) WWP1 specifically ubiquitinates WT but not PY motif-mutated p63 α . (c) Endogenous WWP1 ubiquitinates endogenous Δ Np63 α in MCF10A. WWP1 was knocked down by siRNA#1. Luc siRNA was used as a control. MG132 was not added. IP was performed under a denaturing condition using anti-p63 Ab and protein A beads

measured the steady-state levels of both TAp63 α and Δ Np63 α in the presence and absence of WWP1. As shown in Figure 3a, the steady-state levels of both TAp63 α and Δ Np63 α are decreased in WT WWP1, but not in WWP1C890A, overexpressing LinX cells. To further determine whether the protein interaction between p63 α and WWP1 is required for degradation of p63 α , we measured the steady-state protein level of Δ Np63 α Y449F in the presence of WWP1 or WWP1C890A. As expected, WT WWP1 fails to decrease the steady-state level of Δ Np63 α Y449F (Figure 3a).

To further investigate whether the decrease of p63 α by WWP1 is because of the increase of protein degradation, we measured the half-lives of Δ Np63 α and TAp63 α in the presence and absence of WWP1 by cycloheximide (CHX) chase assays. As shown in Figure 3b, both the Δ Np63 α and TAp63 α proteins have a long half-life (> 10 h) in LinX cells. When WT WWP1 is overexpressed, the half-lives are dramatically decreased to about 3.2 h for Δ Np63 α (Figure 3c) and about 7.5 h for TAp63 α (Figure 3d). The catalytic inactive WWP1C890A only slightly decreases the half-life of Δ Np63 α and TAp63 α when compared with the empty vector (Figure 3c and d). As the PY motif mutant p63 α cannot interact with WWP1, we wondered whether WWP1 cannot decrease the half-lives of these mutant p63 α . Indeed, we found that both Δ Np63 α Y449F and TAp63 α Y543F are resistant to WWP1-mediated degradation (Figure 3b–d).

The degradation of p63 α by WWP1 is most likely through the proteasome as p63 α is polyubiquitinated by WWP1. To test if the proteasome is involved in p63 α degradation by WWP1, we performed CHX chase assays in the presence of the proteasome inhibitor MG132 (10 μ M). As shown in Figure 3(b–d), WWP1-induced p63 α degradation is blocked by MG132, suggesting that the degradation of p63 α by WWP1 is through the 26S proteasome.

We also confirmed that endogenous WWP1 decreases protein half-life of endogenous Δ Np63 α . The WWP1 protein was knocked down by siRNA in MCF10A, the Δ Np63 α protein half-lives were measured by pulse chase assays. As shown in Supplementary Figure S1, WWP1 knockdown dramatically extended the half-life of the endogenous Δ Np63 α protein.

WWP1 targets the endogenous Δ Np63 α protein for degradation and sensitizes immortalized breast epithelial cells to chemotherapeutic drug doxorubicin-induced apoptosis. To determine whether WWP1 targets p63 α under physiological conditions, we knocked down endogenous WWP1 in two immortalized breast epithelial cell lines MCF10A and 184B5 by two different anti-WWP1 siRNAs. We found that the protein levels of endogenous Δ Np63 α , the major p63 isoform in MCF10A and 184B5, are remarkably elevated in both cell lines when WWP1 is knocked down by both siRNAs (Figure 4a). These results

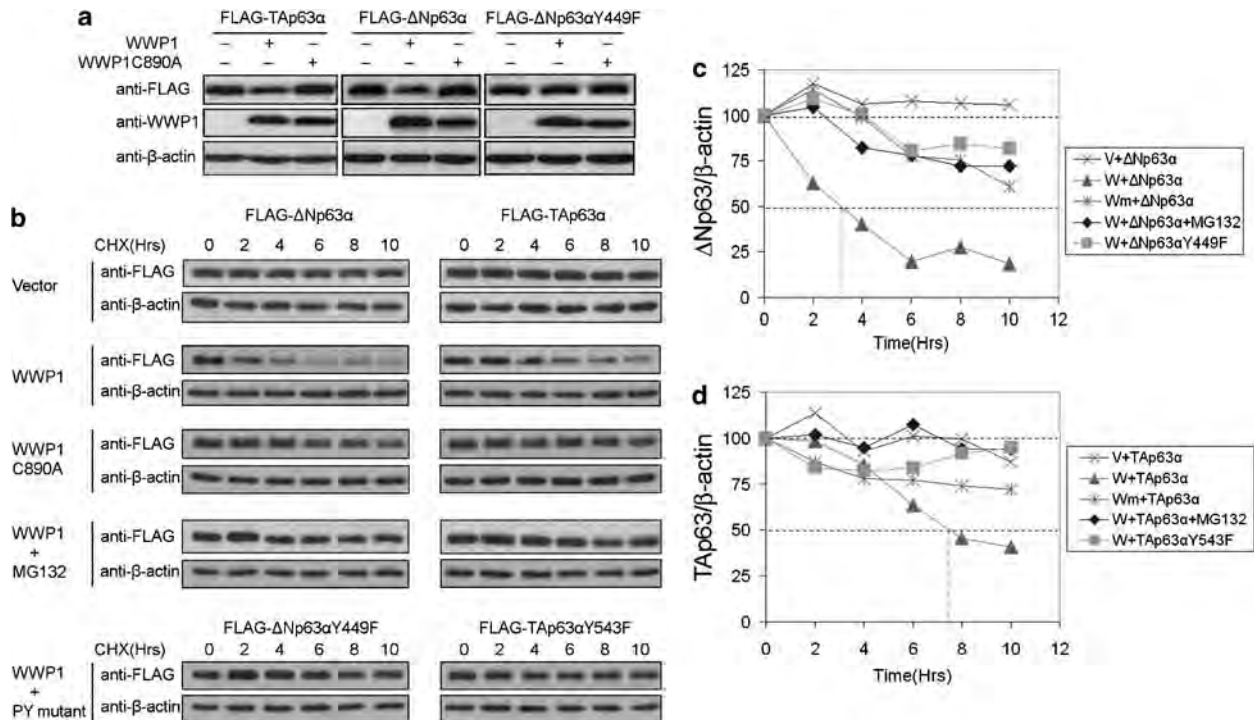


Figure 3 WWP1 promotes proteasomal degradation of p63 α proteins. (a) WT WWP1 decreases the steady levels of both FLAG-TAp63 α and FLAG- Δ Np63 α in LinX cells, as determined by western blot. The PY motif mutant FLAG- Δ Np63 α Y449F is resistant to WWP1-mediated degradation. An empty vector and the catalytic inactive mutant WWP1C890A were used as controls. (b) Measurement of protein half-lives by cycloheximide (CHX) chase assays and western blot. LinX cells were co-transfected with indicated plasmids. Forty-eight hours after transfection, the cells were incubated with 50 μ g/ml CHX for different times (2–10 h) and collected for western blot. β -actin was used as a loading control. MG132 (20 μ M) was added together with CHX as necessary. The exposure times have been adjusted for each panel to compare protein degradation. (c) Quantitative results of Δ Np63 α from panel B by the IMAGE J software. The normalized p63 α at 0 h was defined as 100. V, vector; W, WWP1; Wm, WWP1C890A. (d) Quantitative results of TAp63 α from panel B by the IMAGE J software

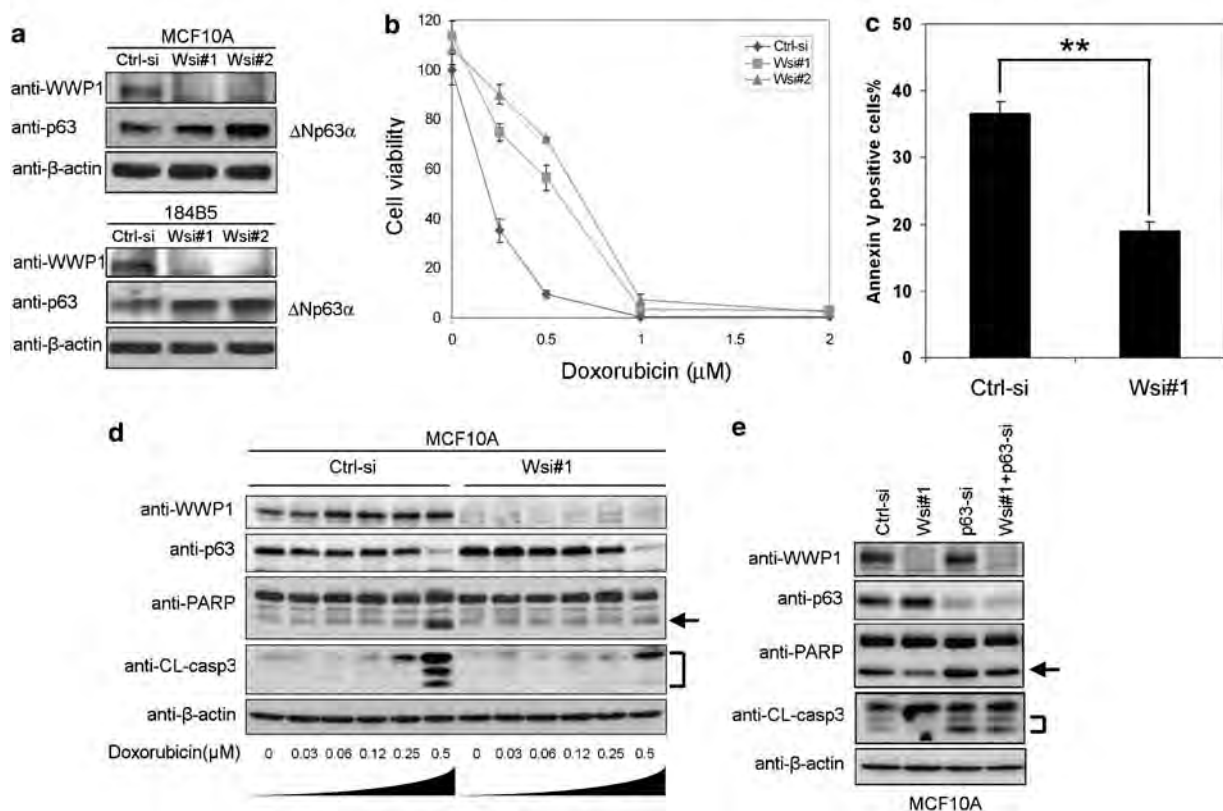


Figure 4 WWP1 siRNA increases the endogenous $\Delta Np63\alpha$ protein and confers resistance to doxorubicin in immortalized breast epithelial cells. (a) The knockdown of WWP1 by two different siRNAs increases the endogenous levels of the $\Delta Np63\alpha$ protein in the MCF10A and 184B5 immortalized breast epithelial cells, as determined by western blot. siRNAs were transfected at 100 nM for 48 h. (b) WWP1 knockdown by siRNAs increases the cell viability in MCF10A cells. Different amounts of doxorubicin were added to cells 48 h after siRNA transfection. The cell viability was analyzed by the SRB assay 2 days later. Error bars mean S.D. (same for all figures in this paper). The data was collected from triplicate samples. The experiments were performed at least two times and similar results were obtained. (c) WWP1 knockdown by WWP1 siRNA#1 significantly decreases the doxorubicin (0.5 μM) induced apoptosis in MCF10A as determined by Annexin V staining. ** $P < 0.01$ (t-test). (d) WWP1 knockdown by WWP1 siRNA#1 decreases the doxorubicin-induced PARP and caspase 3 activation in MCF10A, as determined by Western blot. The cells were treated with doxorubicin for another 24 h after siRNA transfection for 48 h. The cleaved PARP is indicated by an arrow. (e) $\Delta Np63\alpha$ siRNA rescues the WWP1 siRNA-induced doxorubicin resistance in MCF10A. All siRNAs were transfected at 50 nM for 72 h in total. All cells were treated with 0.25 μM doxorubicin for 48 h

suggest that WWP1 targets the endogenous $\Delta Np63\alpha$ protein for degradation.

It has been reported that $\Delta Np63\alpha$ is essential for MCF10A to survive.¹⁹ Therefore, elevation of $\Delta Np63\alpha$ may confer resistance to apoptosis. To test this, we treated siRNA-transfected MCF10A cells with different dosages of doxorubicin for 2 days and measured the cells viability by SRB assays. The high concentration of doxorubicin ($\geq 1 \mu M$) kills all MCF10A cells at the same efficiency (Figure 4b). However, the WWP1 knockdown MCF10A cells are significantly more resistant to low concentrations (0.25–0.5 μM) of doxorubicin than the Ctrl-si-transfected cells. Two different anti-WWP1 siRNAs show similar results although Wsi#2 is slightly more effective than Wsi#1 possibly because of inducing more $\Delta Np63\alpha$ (Figure 4a). We further measured apoptosis by the Annexin V staining. As shown in Figure 4c, Wsi#1 significantly ($P < 0.01$) reduced doxorubicin (0.5 μM) induced Annexin V positive cells. We also confirmed apoptosis by the measurement of the cleaved PARP and caspase3 levels, two typical molecular markers for apoptosis. As shown in Figure 4d, the cleaved PARP and caspase3 are induced by doxorubicin (0.25–0.5 μM) in Ctrl-si-transfected MCF10A. In contrast, in

Wsi#1-transfected MCF10A cells, the cleaved PARP and caspase3 are either undetectable or at low levels after treatment of doxorubicin (0.25–0.5 μM). Consistently, we noticed that the $\Delta Np63\alpha$ protein levels are elevated in Wsi#1-transfected MCF10A cells without or with low concentration of doxorubicin. However, doxorubicin decreases the $\Delta Np63\alpha$ protein levels in a dosage-dependent and WWP1-independent manner. The exact mechanism of $\Delta Np63\alpha$ degradation by doxorubicin is currently unknown.

To further test whether WWP1 regulates apoptosis through targeting $\Delta Np63\alpha$ for degradation, we knocked down both WWP1 and $\Delta Np63\alpha$ in MCF10A and treated the cells with 0.25 μM doxorubicin (Figure 4e). Consistent with the previous results, the WWP1 siRNA-transfected MCF10A cells show a higher level of $\Delta Np63\alpha$ and lower levels of cleaved PARP and cleaved caspase 3 than the Ctrl-si-transfected MCF10A cells. As reported,²⁴ knockdown of $\Delta Np63\alpha$ alone increases the levels of cleaved PARP and cleaved caspase 3. More importantly, knockdown of $\Delta Np63\alpha$ abolishes the WWP1 siRNA-induced $\Delta Np63\alpha$ increase and drug resistance. We also confirmed these results by the SRB assay (data not shown). These findings suggest that WWP1 regulates

apoptosis and drug resistance in MCF10A through targeting Δ Np63 α for degradation.

WWP1 targets the endogenous TAp63 α protein for degradation and confers cell survival in a p53-independent manner. Based on the results in Figure 1–3, WWP1 targets not only Δ Np63 α but also TAp63 α for degradation. Numerous studies have shown that the endogenous TAp63 α protein is usually at low levels in epithelial cells.¹⁶ In a recent study, the endogenous TAp63 α isoform is expressed in the HCT116 colon cancer cell line.²⁷ We validated the expression of TAp63 α in Figure 1e. Therefore, we sought to test whether WWP1 targets endogenous TAp63 α in HCT116 cells and regulates apoptosis. As shown in Figure 5a, we efficiently knocked down WWP1 in HCT116 using two different anti-WWP1 siRNAs. As expected, the endogenous TAp63 α levels are

elevated in WWP1 knockdown cells. WWP1 siRNA#2 causes a more significant increase of TAp63 α than siRNA#1 possibly because of different knockdown efficiency in this cell line. Then we measured apoptosis by PARP cleavage. Consistent with the elevated TAp63 α levels, the cleaved PARP protein level is increased in the WWP1 knockdown HCT116 cells. We confirmed this result by measuring cell viability with SRB assays. We found that both anti-WWP1 siRNAs significantly decrease HCT116 cell viability (Figure 5b). Consistent with the higher levels of TAp63 α and cleaved PARP, siRNA#2 more effectively decreases cell survival than siRNA#1. WWP1 has been shown to suppress p53 function.¹³ To further determine whether WWP1 regulates apoptosis through p53 rather than TAp63 α , we performed the same experiment in p53-null HCT116 cells. There is no significant difference between the p53-null HCT116 cells and the p53 WT HCT116 cells in

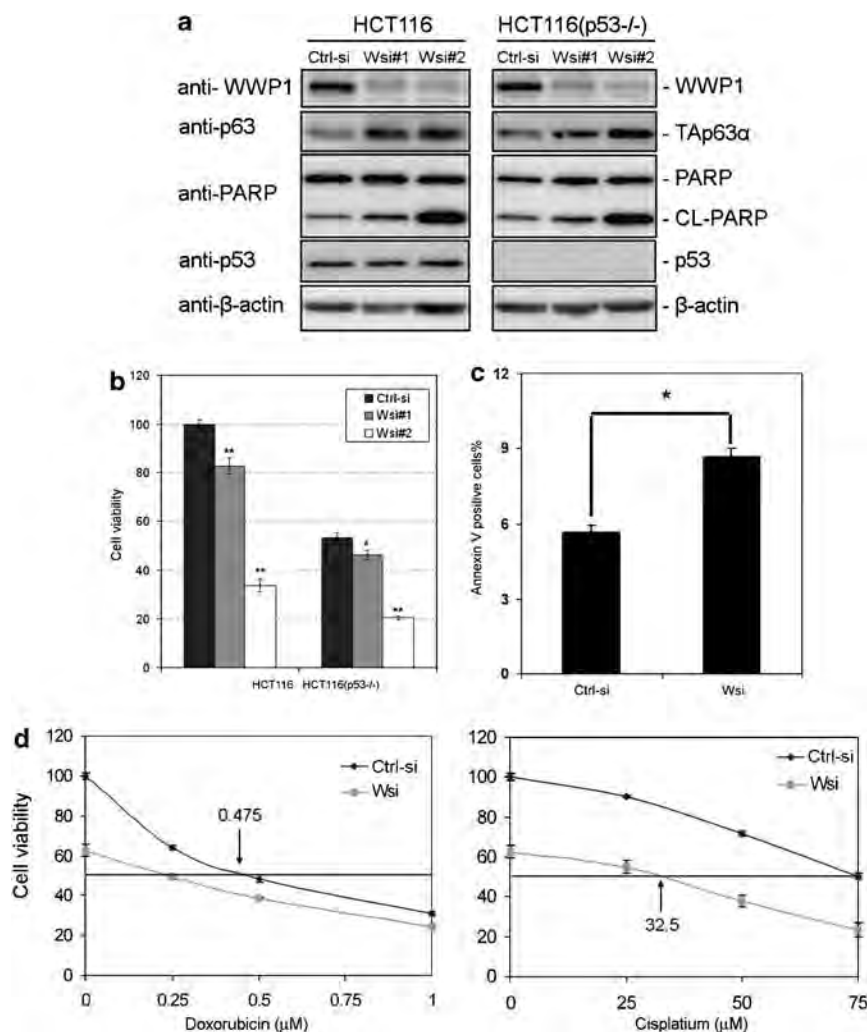


Figure 5 WWP1 knockdown by siRNAs upregulates the endogenous TAp63 α levels and induces apoptosis in a p53-independent manner in HCT116 cells. (a) The knockdown of WWP1 by two different siRNAs increases the levels of the endogenous TAp63 α protein and the cleaved PARP protein in the HCT116 and HCT116 (p53-/-) colon cancer cell lines. siRNAs were transfected at 100 nM for 48 h. (b) The knockdown of WWP1 by two different siRNAs decreases cell viability in the HCT116 and HCT116 (p53-/-) colon cancer cell lines. The cell viability was analyzed by the SRB assay after transfection with WWP1 siRNA for 72 h. * $P < 0.05$; ** $P < 0.01$ (t -test). (c) WWP1 knockdown by WWP1 siRNA#2 significantly induces apoptosis in HCT116, as determined by Annexin V staining. * $P < 0.05$ (t -test). (d) The knockdown of WWP1 in HCT116 cells decreases the IC₅₀ for doxorubicin and cisplatin. The cell viability was analyzed by the SRB assay after transfection with WWP1 siRNA for 72 h in total and treatment with drugs for 48 h. The data was collected from triplicate samples. The experiments were performed at least two times and similar results were obtained.

terms of the induction of Tap63 α , cleaved PARP and apoptosis by WWP1 siRNA. We noticed that WWP1 siRNA may decrease cell viability slightly more effective in p53 WT HCT116 cells than in p53-null cells. However, the p53 levels are not significantly changed by WWP1 knockdown (Figure 5a). Furthermore, we measured apoptosis by Annexin V staining and found that WWP1 siRNA significantly increases Annexin V positive HCT116 cells (Figure 5c). These findings indicate that WWP1 siRNAs induce Tap63 α and apoptosis in a p53-independent manner.

To study the biological relevance of WWP1-mediated degradation of Tap63 α , we transfected WT Tap63 α and the stable PY motif-mutated Tap63 α Y543F into HCT116 (p53 $^{-/-}$) cells and examined the proliferation/apoptosis index by SRB assays. We found that overexpression of Tap63 α enhances cleaved caspase 3 and reduces cell viability when compared with the empty vector control (Supplementary Figure S2). Consistent with the protein stability, the level of Tap63 α Y543F is higher than that of WT Tap63 α . Compared with WT Tap63 α , Tap63 α Y543F induces more caspase 3 cleavages and cell viability loss.

As WWP1 siRNA induces apoptosis in both HCT116 WT and p53-null cells, we further tested whether inhibition of WWP1 sensitizes cells to chemotherapeutic drugs. The HCT116 cells were transfected with Ctrl-siRNA or WWP1 siRNA for 1 day and treated with different concentration of doxorubicin or cisplatin for 2 days. As shown in Figure 5c, WWP1 siRNA and both chemotherapeutic drugs additively decrease the cell viability. After combination with WWP1

siRNA, the half maximal inhibitory concentration (IC₅₀) for doxorubicin is decreased from 0.475 to 0.25 μ M and the IC₅₀ for cisplatin is decreased from 75 to 32.5 μ M. Similar results were obtained in HCT116 p53-null cells (data not shown).

DNA damage chemotherapeutic drugs induces WWP1 expression in a p53-dependent manner. We noticed that the WWP1 protein is induced by doxorubicin in a dosage-dependent manner in MCF10A (Figure 4d). We wondered whether WWP1 is also induced by doxorubicin in HCT116 cells. To test this, the WT and p53-null HCT116 cells were treated with 1 μ M doxorubicin at different times. As a result, doxorubicin induces both p53 and WWP1 in WT HCT116 cells in a time-dependent manner (Figure 6a). The induction peak for WWP1 is 48 h. Surprisingly, doxorubicin does not induce WWP1 in p53-null HCT116 cells. These results were confirmed at mRNA levels by qRT-PCR (Figure 6b). These results indicate that doxorubicin may induce WWP1 transcription in a p53-dependent manner.

To further test whether other DNA damage chemotherapeutic drugs also induce WWP1 in a p53-dependent manner, we treated the WT and p53-null HCT116 cells with different dosage of doxorubicin, cisplatin and etoposide. As shown in Figure 6c and d, both doxorubicin and cisplatin induce WWP1 in WT but not p53-null HCT116 cells in a dose-dependent manner. Similar results were observed with etoposide treatment (data not shown). These findings suggest that DNA damage chemotherapeutic drugs can induce the WWP1 expression in a p53-dependent manner. To further confirm

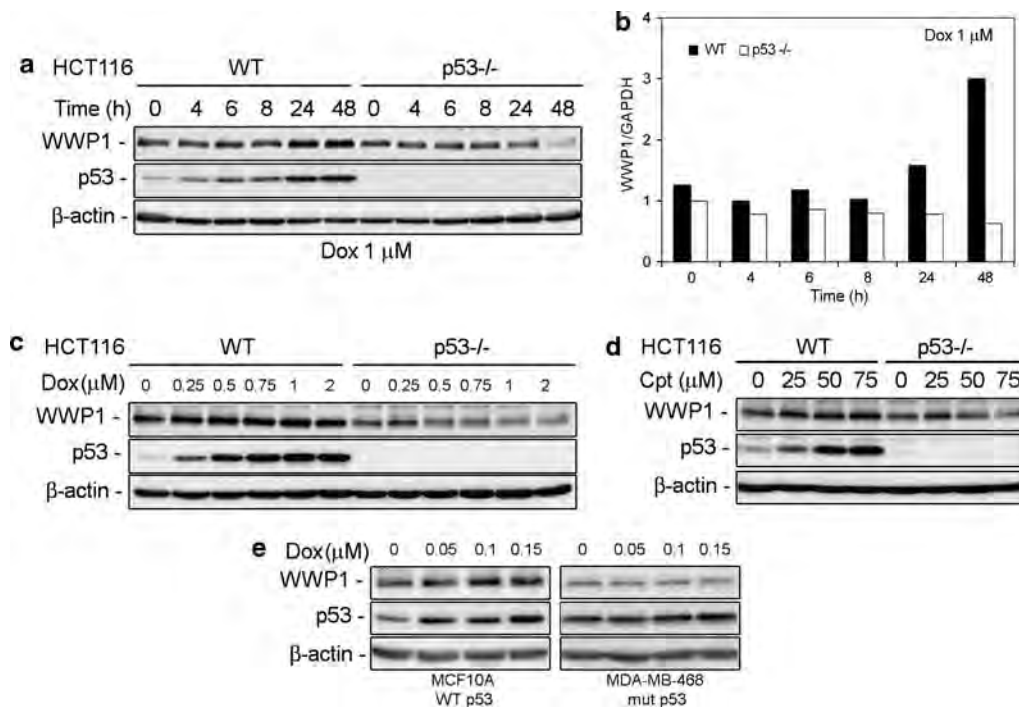


Figure 6 DNA damage drugs induce the WWP1 mRNA and protein in a p53-dependent manner. (a) The WWP1 protein is induced by doxorubicin (Dox, 1 μ M) in a time-dependent manner in WT but not p53-null HCT116 cells. The WWP1, p53 and β -actin proteins were detected by western blot. (b) The WWP1 mRNA is induced by doxorubicin (Dox, 1 μ M) in a time-dependent manner in WT but not p53-null HCT116 cells. The WWP1 and GAPDH mRNA were detected with qRT-PCR.³ (c) The WWP1 protein is induced by doxorubicin in a dose-dependent manner in WT but not p53-null HCT116 cells. (d) The WWP1 protein is induced by cisplatin (Cpt) in a dose-dependent manner in WT but not p53-null HCT116 cells. (e) The WWP1 protein is induced by doxorubicin in a dose-dependent manner in MCF10A (WT p53) but not in MDA-MB-468 (mutant p53) cells

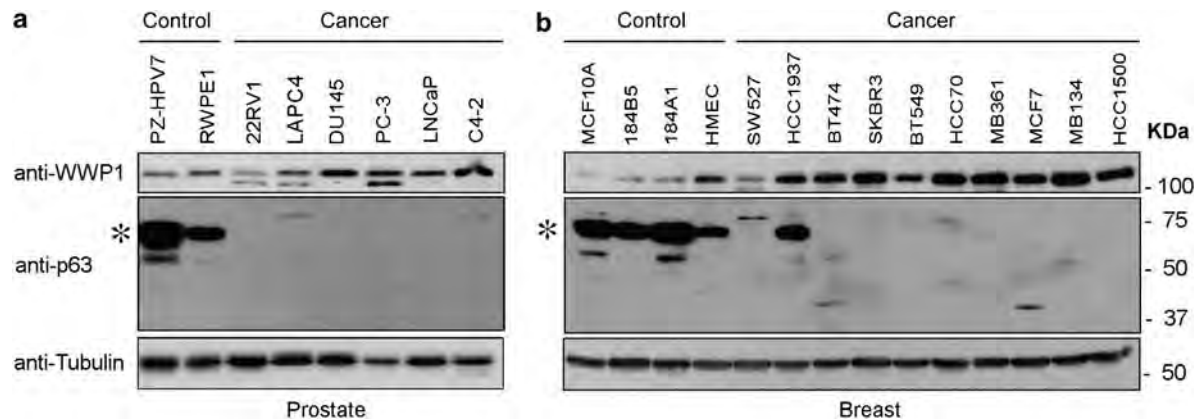


Figure 7 Expression of the WWP1 and p63 proteins in prostate and breast cell lines. Western blots were performed in a panel of prostate (a) and breast (b) cell lines to determine the protein expression levels of WWP1 and p63. The controls are immortalized cell lines. The molecular weight markers are labeled at the right. Tubulin was used as a loading control. The protein expression level of WWP1 is negatively correlated with the protein expression level of p63 α , especially Δ Np63 α (*) in these samples

these results in other cell lines other than HCT116, we treated MCF10A (WT p53)²⁸ and MDA-MB-468 (mutant p53)²⁹ with different concentration of doxorubicin for 48 h and found that WWP1 is only induced in MCF10A but not in MDA-MB-468 although p53 is accumulated in both cell lines (Figure 6e).

The protein expression of WWP1 and p63 is reversely correlated in prostate and breast cancer cell lines. The p63 protein has been documented to be specifically expressed in normal basal epithelial cells but lost in cancer cells from the prostate and breast.^{30,31} In sharp contrast, WWP1 is frequently overexpressed in prostate and breast cancer cells. As WWP1 targets p63 for degradation, we asked whether the protein expression of WWP1 is negatively correlated with the protein expression of p63 in prostate and breast cancers.

We examined the protein expression levels of WWP1 and p63 in a panel of prostate and breast cell lines by western blot (Figure 7a–b). Consistent with our previous report,² the protein levels of WWP1 are elevated in four prostate cancer cell lines LAPC-4, DU145, PC-3 and LNCaP/C4-2 compared with two immortalized prostate cell lines PZ-HPV7 and RWPE1 (Figure 7a). The protein expression of p63 (mainly Δ Np63 α isoform) is negatively correlated with the expression of WWP1 in all these cell lines. The only exception is 22Rv1, in which the expression of WWP1 is not increased but p63 is still undetectable. Similar results are obtained in 10 breast cancer cell lines and four immortalized breast cell lines (Figure 7b). The only exception is HCC1937, in which the expression of WWP1 is increased but the Δ Np63 is still highly expressed. Therefore, we conclude that there is a negative correlation between WWP1 and p63 in a majority of prostate and breast cancer cell lines.

Discussion

In this study, we provide several lines of evidence to support that WWP1 targets the p63 protein for ubiquitin-mediated proteasomal degradation. First, WWP1 binds to p63 α proteins through the WW/PY motif interaction. Second, WWP1 ubiquitinates p63 α through its E3 ligase activity. Third,

WWP1 promotes p63 α for proteasomal degradation. In addition, the WWP1 siRNA upregulates the endogenous Δ Np63 α and TAp63 α protein levels. Finally, there is a negative correlation between the WWP1 and p63 proteins in prostate and breast cells.

It is well documented that p63 proteins have important functions in epithelial development and tumorigenesis through regulating epithelial progenitor cell proliferation, differentiation and apoptosis.^{17,18} p63 proteins are expressed in the basal cells of normal prostate glands but not in prostatic carcinomas.³⁰ Similarly, the expression of p63 proteins is in the nuclei of myoepithelial cells of normal breast ducts and lobules but not in invasive breast cancer except metaplastic carcinomas.³² Several mechanisms including transcription regulation²⁰ and post-translational modification contribute to the loss of p63 protein expression in prostate and breast cancers. Increasing evidence suggests that protein ubiquitination and degradation have an important function for the p63 activity.^{23–25,33} Two other WWP1 family members, Itch and Nedd4, have been previously reported to target p63 proteins for proteasomal degradation.^{24,25} However, the expression levels of Itch in breast cancer are not altered³⁴ although the expression of Nedd4 has been found to be overexpressed in invasive bladder cancer cells.⁴ WWP1 is amplified and overexpressed in more than 30% of prostate and breast cancers, suggesting that WWP1 among these E3 ligases may have a major function for the p63 protein degradation in prostate and breast cancers, although we cannot completely exclude the roles of Itch and Nedd4. Under the physiological conditions, how WWP1, Itch and Nedd4 are coordinately activated and specially recruit p63 need further investigation.

Importantly, different p63 isoforms have different biological functions. There are at least six isoforms of p63, α , β and γ (each including TAp63 and Δ Np63). Numerous studies suggest that TAp63 isoforms induce apoptosis but Δ Np63 isoforms inhibit apoptosis. However, the functions of Δ Np63 from different reports are not consistent and sometimes even contradictory. Δ Np63 is essential for cancer cell survival through inhibiting TAp63 in squamous cell carcinoma (SCC)³⁵ and a subset of breast cancer with p53 mutation.²⁰ Δ Np63 has been reported to be overexpressed in primary SCCs of the

head and neck.³⁶ Thus, it would be interesting to examine whether WWP1 is frequently downregulated in SCCs. However, Δ Np63 α is proposed to act as a metastasis suppressor by maintaining the epithelial phenotype of cancer cells.³⁷ Loss of Δ Np63 has been reported to decrease epithelial cell adhesion and promote cell migration.¹⁹ Furthermore, Δ Np63 has even been demonstrated to possess a growth suppression function.³⁸ p63 is frequently lost in most invasive adenocarcinomas including prostate cancer,³⁰ breast cancer,³¹ bladder cancer³⁹ and lung cancer.⁴⁰ Therefore, the function of p63 may be context-dependent in different cancers.

It is well established that the functions of p63 depend on the expression pattern of p53 family members. In breast carcinomas, Δ Np63 isoforms are co-expressed with TAp73 exclusively within a subset of triple-negative (ER/EGFR/HER2 negative) primary breast cancers that commonly exhibit mutational inactivation of p53.²⁰ We demonstrated that WWP1 regulates both Δ Np63 α and TAp63 α isoforms in this study. WWP1 may target the β isoforms but not the γ isoforms because both α and β , but not γ , have a PY motif. However, WWP1 has been reported to suppress the function of p53 through nuclear export although p53 does not have a PY motif.¹³ We found that WWP1 does not affect p53 stability in HCT116 and that WWP1 promotes p63 degradation independent of the p53 status (Figure 5a). In addition, Itch has been reported to target p73 for ubiquitin-mediated degradation because p73 contains PY motifs.⁴¹ Similarly, we found that WWP1 can also ubiquitinate p73 and decrease the stable level of p73 (unpublished observation). Thus, the functional output of WWP1 may rely on the expression pattern of p53 family members.

The expression pattern of p63 isoforms and p53 family members are different and complicated in different cancer cells. We found that WWP1 targets both Δ Np63 α and TAp63 α for degradation, implicating that WWP1 has a context-dependent role in terms of apoptosis and tumor development. Indeed, WWP1 sensitizes the MCF10A immortalized breast epithelial cells to doxorubicin-induced apoptosis but increases the HCT116 colon cancer cell survival and drug resistance. WWP1 is frequently amplified and overexpressed in prostate and breast cancers, suggesting that WWP1 overexpression may increase cell survival. In agreement with this idea, WWP1 knockdown in MCF7 and HCC1500 breast cancer cell lines causes apoptosis.³ We do not know whether the WWP1 ablation-induced apoptosis in these cells is actually through the increase of TAp63 because we did not detect the increase of TAp63 by western blot (data not shown). However, we cannot exclude that TAp63 has a function because TAp63 isoforms can be transcriptionally active at the levels below the limit of detection by western blot.⁴² Similarly, we cannot exclude that WWP1 promotes HCT116 cell survival through other molecules other than TAp63. The role and mechanistic action of WWP1 in cancer still need to be elucidated *in vivo* using transgenic mouse models.

WWP1 is amplified and overexpressed in over 40% of breast cancers and confers an advantage for cancer cell survival. In contrast, Δ Np63 is frequently lost in breast cancer cells. Apparently, the survival of the breast cancer cells with WWP1 overexpression does not depend on Δ Np63 α . Only a

small percentage of triple-negative breast cancers depend on Δ Np63 because TAp73 is overexpressed and p53 is mutated.²⁰ If WWP1 inhibits TAp73 in these cells, the cell survival would not require Δ Np63 any longer. Therefore, we would predict that WWP1 expression is very low in triple-negative breast cancer cells. Indeed, WWP1 is preferably overexpressed in ER positive and p53 WT breast cancers (www.Oncomine.org). The immunohistochemical staining results in invasive breast tumors confirmed that the expression of WWP1 associates with positive ER status (Chen *et al.*, manuscript submitted). Therefore, the expression of WWP1 may be invaluable for breast cancer diagnosis and prognosis.

The transcriptional regulation of WWP1 is largely unknown although we have previously shown that WWP1 is induced by TGF β as a negative feedback mechanism.¹ Laine *et al.*,¹³ reported that UV-irradiation or γ -irradiation decreases WWP1 mRNA expression in mouse embryonic fibroblasts in a p53-dependent manner. However, we found that several DNA damage chemotherapeutic drugs including doxorubicin, cisplatin and etoposide induces the WWP1 expression in HCT116 and breast cell lines in a p53-dependent manner. These conflicting results may be caused by different treatments in different cells. Whether WWP1 is an Mdm2 like p53 direct target gene needs elucidation in future studies.

In summary, we demonstrate that WWP1 targets p63 proteins for ubiquitin-mediated proteasomal degradation. We show that WWP1 plays opposite roles in terms of apoptosis and drug sensitivity in immortalized cells and cancer cells depending on the expression of different p63 isoforms. Given the frequent gene amplification and overexpression of WWP1 in prostate and breast cancers, these findings may help us understand the role of WWP1 in cancer development and may provide rationale to develop WWP1 as a diagnosis marker and molecular target for cancer therapy.

Materials and Methods

Cell culture and transfection. All breast and prostate cell lines have been described in our previous studies.^{3,43,44} The human embryonic kidney 293T-derived LinX cell line was cultured in DMEM containing 5% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PS). The p53 wild type (WT) and p53-null HCT116 colon cancer cell lines were grown in HyQ[®] McCoy's 5A medium with 5% FBS and 1% PS. All transient transfection for plasmids and siRNAs was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. All chemically synthesized siRNAs were purchased from Dharmacon (Chicago, IL, USA) and transfected at 100 nM final concentration. The siRNA target sequences for human WWP1 gene are 5'-GAAGTCA TCTGTAACATAA-3' (Wsi#1) and 5'-GCAGAGAAATACTGTTAT-3' (Wsi#2). The target sequence for Δ Np63 α is 5'-CAGGTTGGCACTGAATTCACG-3'. The 3'-UU overhang for both strands and 5'-phosphorylation for anti-sense sequence were employed for these siRNAs.

Expression plasmids. The plasmids expressing WT WWP1, the catalytic inactive hWWP1C890A, and mWWP1C886S have been described in our previous studies.^{3,12} The Myc-Ub construct pCMV-6XHis-Myc-Ub is a gift from Dr. Raymond J. Deshaies (California Institute of Technology). The pcDNA3-FLAG-TAp63 α and pcDNA3-FLAG- Δ Np63 α constructs were obtained by subcloning the TAp63 α and Δ Np63 α cDNA into the *Hind* III and *Not* I sites of pcDNA3. The PY motif-mutated FLAG-TAp63 α Y543F and FLAG- Δ Np63 α Y449F were obtained by using a QuikChange[®] site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Constructs for GST-WW domains were obtained by subcloning the DNA fragments encoding the WW domains of hWWP1 into the *Bam*HI and *Not*I sites of the pEBG vector.

Immunoprecipitation and GST pull down. Immunoprecipitation using an anti-Myc antibody (Ab) plus protein A-agarose beads and GST pull down using the glutathione-Sepharose 4B slurry beads have been described in a previous study.¹² Briefly, LinX cells were transfected with expression plasmids in 60-mm culture dishes for 48 h. The cells from each dish were collected into 0.6 ml of 1 × ice-cold cell lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% protease inhibitor cocktail (no. P8340, Sigma, St. Louis, MO, USA)) and incubated on ice for 30 min. Then, cell lysates were centrifuged at 10 000 g for 10 min at 4°C. The supernatant (200 µl) and primary anti-Myc antibody (2 µl) were incubated with gentle rocking overnight at 4°C. In all 30 µl of 50% protein A-agarose beads were added and incubated for 1–3 h at 4°C. The anti-WWP1 mouse monoclonal Ab (1A7) was used to replace anti-Myc Ab to immunoprecipitate the endogenous WWP1 proteins from MCF10A. The mouse IgG was used as the negative control. For the GST pull-down assay, the glutathione-Sepharose 4B slurry beads were directly incubated with the supernatant overnight at 4°C. The beads were washed five times with 500 µl of 1 × cell lysis buffer. Proteins were resuspended with 20–50 µl of SDS sample buffer and analyzed by western blot.

Antibodies and western blot. The anti-WWP1 rabbit polyclonal antibody (Ab) has been described in our previous report (3). The anti-WWP1 mouse monoclonal Ab (1A7) is from Novus Biologicals Inc. (Littleton, CO, USA). The anti-β-actin mouse monoclonal Ab AC-15 (no. A5441), the anti-FLAG rabbit polyclonal Ab (no. F7425), the anti-GST rabbit polyclonal Ab (no. G7781) are from Sigma. The anti-p63 mouse monoclonal Ab 4A4 (no. Sc-8431), anti-Myc mouse monoclonal Ab 9B11 (no. 2276), anti-PARP (no. 9915), anti-caspase 3 (no. 9915), and anti-tubulin antibodies (no. 2148) are from Cell Signaling (Danvers, MA, USA). The anti-p53 mouse monoclonal Ab (no. 554169) is from BD Pharmingen (San Diego, CA, USA).

Proteins were separated by SDS-PAGE and blotted onto PVDF membranes. The membranes were blocked with PBST (PBS with 0.1% Tween 20) buffer containing 5% nonfat dry milk and incubated with primary antibodies diluted in PBST overnight at 4°C. After washing three times in PBST, the membranes were incubated with 10 000 times diluted horseradish peroxidase-conjugated second antibodies for 1 h at room temperature. Detection was performed with the Supersignal West Pico enhanced chemiluminescence system (Pierce, Rockford, IL, USA) and a LAS-3000 Fujifilm imaging system.

Protein ubiquitination assay. LinX cells were transiently transfected with Myc-Ub and other plasmids as necessary in 6-well plates. Two days after transfection, the cells were harvested in 150 µl SDS lysis buffer (50 mM Tris-Cl, pH 6.8, 1.5% SDS). The samples were boiled for 15 min. 100 µl of protein lysate was diluted with 1.2 ml EBC/BSA buffer (50 mM Tris-Cl, pH 6.8, 180 mM NaCl, 0.5% CA630, 0.5% BSA) and incubated with 30 µl 50% anti-FLAG[®] M2-agarose beads (no. A2220, Sigma) overnight at 4°C with rotation. The beads were collected by centrifugation at 10 000 g for 30 s at 4°C and washed three times with 1 ml ice-cold EBC/BSA buffer. Proteins were resuspended with 30 µl of SDS sample buffer and analyzed by western blot. Ub-conjugated p63 was detected by anti-Myc Ab. For the endogenous p63 ubiquitination, IP was performed by using anti-p63 Ab; and ubiquitinated p63 was detected by anti-Ub Ab.

Measurement of apoptosis. The siRNA-transfected MCF10A and HCT116 cells were exposed to different concentrations of doxorubicin (Sigma) or Cisplatin (Cpt) at different times. The cell viability was measured by the SRB assay and the Annexin V staining as described in our previous report.² The protein levels of cleaved PARP and/or cleaved caspase 3 were used to measure apoptosis.

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Overexpression of WWP1 is associated with the estrogen receptor and insulin-like growth factor receptor 1 in breast carcinoma

Ceshi Chen^{1*}, Zhongmei Zhou¹, Christine E. Sheehan², Elzbieta Slodkowska², Christopher B. Sheehan², Ann Boguniewicz² and Jeffrey S. Ross^{2*}

¹The Center for Cell Biology and Cancer Research, Albany Medical College, Albany, NY

²The Department of Pathology and Laboratory Medicine, Albany Medical College, Albany, NY

WWP1, a HECT type E3 ubiquitin ligase frequently amplified and overexpressed in breast cancer, has the potential to become a useful clinical biomarker and therapeutic target in breast cancer. Here, we performed immunohistochemical staining in formalin-fixed and paraffin-embedded tissue sections from 187 cases of primary invasive mammary carcinoma [137 ductal carcinomas (IDC) and 50 lobular carcinomas (ILC)] by using a monoclonal anti-WWP1 antibody. The normal breast epithelium and adjacent benign epithelium are essentially negative for WWP1. Cytoplasmic WWP1 immunoreactivity was observed in 76/187 (40.6%) tumors and showed a positive correlation with ER α ($p = 0.05$) and IGF-1R proteins ($p = 0.001$) in this cohort. The positive correlations between WWP1 and ER/IGF-1R were also observed in a panel of 12 breast cancer cell lines by Western blot. Interestingly, the ER levels are decreased when WWP1 is silenced in ER positive MCF7 and T47D breast cancer cell lines. Finally, WWP1 ablation collectively inhibits cell proliferation with tamoxifen in MCF7 and T47D, as measured by ³H-thymidine incorporation assays. These findings suggest that WWP1 may play an important role in ER positive breast cancer.

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Key words: WWP1; breast cancer; IHC; ER; IGF-1R

The WW domain containing the E3 ubiquitin protein ligase 1 (WWP1) belongs to the C2-WW-HECT type E3 ubiquitin ligase family, which comprises 9 members including NEDD4, AIP4/Itch, SMURF1 and SMURF2.^{1,2} Four family members including WWP1,^{3,4} NEDD4,⁵ SMURF2⁶ and SMURF1⁷ have been shown to be overexpressed in different tumor types.² WWP1 regulates multiple important proteins involved in tumorigenesis. Several studies suggest that WWP1 negatively regulates the transforming growth factor- β (TGF- β) signaling by targeting its molecular components, including TGF- β receptor 1 (T β R1),⁸ Smad2⁹ and Smad4,¹⁰ for ubiquitin mediated degradation. In addition, WWP1 has been reported to regulate the expression levels of the epithelial Na⁺ channel (ENaC)¹¹ Notch,¹² EGFR/ErbB2,¹³ Runx2,^{14,15} KLF2,¹⁶ KLF5,¹⁷ p53¹⁸ and p63.¹⁹

WWP1 is a potential oncogene that undergoes genomic amplification and overexpression in human breast and prostate cancer.^{3,4} Functionally, WWP1 knockdown significantly suppresses cell proliferation and/or induces apoptosis.^{3,4} It has been previously noticed that WWP1 mRNA is more frequently up-regulated in estrogen receptor (ER) α positive breast cancer cell lines.⁴ This concept is further supported by 8 independent microarray studies in which the WWP1 mRNA levels are universally higher in ER+ compared with ER-breast tumors (Oncomine.org). ER has long been recognized to play an important role in breast cancer initiation; and a majority of human breast tumors are ER positive tumors that can be treated with hormonal-based therapeutics such as tamoxifen, fulvestrant and aromatase inhibitors.²⁰ Thus, WWP1 has the potential to become a useful clinical biomarker and a potential stand-alone prognostic factor.²¹

The insulin-like growth factor 1 receptor (IGF-1R) is a tyrosine kinase growth factor receptor that has been linked to prognosis in a variety of malignancies. IGF-1R is a tetramer composed of 2 extracellular α subunits (130 kDa) and 2 transmembrane β subunits (90 kDa). Activation of IGF-1R by autocrine, paracrine and endocrine stimulation via exposure to its activating ligand, the

IGF-1, leads to cell proliferation and survival.²² As expected, MMTV-IGF-1R Tg mice develop mammary tumors with a short latency.^{23,24} In addition, the protein expression of IGF-1R has been reported to be upregulated in breast cancer.²⁵ Interestingly, the expression of IGF-1R is correlated with ER in primary breast cancer²⁶ and is downregulated in advanced breast cancer.²⁷ The high expression of IGF-1R mRNA in breast cancer tissues has been associated with a more favorable overall and disease-free survival.²⁸ However, the overexpression of IGF-1R in breast tumors and the correlation between the IGF-1R overexpression and prognosis or other clinicopathologic parameters cannot be confirmed in other studies.^{29,30} It is worth pointing out that the ligand induced IGF-1R degradation may be regulated by Nedd4, a WWP1 family member.³¹

The aim of this study is to test whether the WWP1 protein expression in breast tumors is a good prognosis marker and WWP1 is a potential therapeutic target for breast cancer. Here, we found that the WWP1 protein expression is associated with ER and IGF-1R in human breast cancer. Furthermore, inhibition of WWP1 in combination with tamoxifen may be used to treat ER positive breast cancer.

Material and methods

Specimens and clinicopathologic variables

One hundred eighty-seven patients who underwent either a mastectomy or local excision for primary invasive mammary carcinoma between 1983 and 1998 at the Albany Medical Center Hospital were randomly selected. This study was approved by the Albany Medical Center Institutional Review Board. The clinical and pathologic records, tissue blocks and hematoxylin and eosin stained slides were retrieved for each case. Slides were reviewed and blocks were identified based on the presence of adequate tumors and the representative nature of the overall tumor. The tumor type, age at diagnosis, lymph node (LN) status, tumor size, tumor grade, pathologic stage, estrogen and progesterone steroid hormone receptor status, HER-2/neu status, recurrence and overall survival were obtained by review of the medical records. The estrogen/progesterone receptor (ER/PR) measured by competitive binding assays and immunohistochemistry (IHC) was available for 179 (96%) and 166 (89%) cases, respectively. There were 180

Abbreviations: Ab, antibody; EGFR, epithelial growth factor receptor; ER, estrogen receptor; HECT, homologous to the E6-associated protein carboxyl terminus; IGF-1R, insulin growth factor 1 receptor; IHC, immunohistochemistry; siRNA, short interfering RNA; WWP1, WW domain containing E3 ubiquitin protein ligase 1.

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*Correspondence to: The Center for Cell Biology and Cancer Research, Albany Medical College, 47 New Scotland Ave., Albany, NY, 12208, USA. E-mail: chenc@mail.amc.edu or The Department of Pathology and Laboratory Medicine, Albany Medical College, 47 New Scotland Ave., Albany, NY, 12208, USA. E-mail: rossj@mail.amc.edu

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(96%) cases with either HER-2/neu protein status determined by IHC or HER-2/neu gene amplification status determined by fluorescence in situ hybridization (FISH), or by both. One hundred thirty-seven (73%) tumors are invasive ductal carcinoma and 50 (27%) are invasive lobular carcinoma. The patients ranged in age from 26 to 89 years with a mean age of 58 years at diagnosis. Menopausal status was considered by defining premenopausal age as < 45 years, perimenopausal age as 45 to 55 years and postmenopausal age as > 55 years. LN status was available for 179 (96%) cases. Tumor size was available for 176 (94%) cases. One hundred thirty-nine (74%) tumors were graded using the modified Bloom and Richardson method. All cases were staged at the time of diagnosis according to the American Joint Committee on Cancer criteria using the TNM classification scheme. The overall survival status was available for 185 cases (99%) and recurrence data for 183 (98%) cases. The patients were followed for a mean of 97 months (range 1–202 months). On multivariate analysis, early age at diagnosis, large tumor size, advanced stage, node positive status, HER2 positive status and disease recurrence predicted reduced overall survival.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections from a representative block of each of the 187 cases of primary invasive mammary carcinoma were immunostained by automated methods using an indirect biotin avidin diaminobenzidine (DAB) detection system (Ventana Medical Systems, Tucson, AZ). Briefly, sections were dewaxed and rehydrated. No unmasking of the antigenic determinant sites was required for WWP1 staining. For IGF-1R, antigens were retrieved by microwave in EDTA buffer for 22 min at 98°C. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. The mouse anti-human monoclonal WWP1 primary antibody (Novus Biologicals, Littleton, CO) was used at a dilution of 1:100 and the mouse anti-human IGF-1R antibody (sc-462; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:10 for 32 min at 37°C. The secondary antibody for both primaries was biotinylated goat anti-mouse immunoglobulins. After incubation with a streptavidin enzyme conjugate, the complex was then visualized by development of the color with DAB and the slides were counterstained with hematoxylin. WWP1 staining was first optimized and validated using formalin-fixed, paraffin-embedded sections from MCF10A and MCF7 cellblocks. Similarly, processed sections from human colon adenocarcinoma were used as positive controls for IGF-1R immunostaining. To confirm the specificity of both of the primary antibodies, negative control slides were run with every batch, using a predilute negative control reagent (Ventana).

Staining interpretation

Immunoreactivity for both WWP1 and IGF-1R was interpreted by 2 pathologists without prior knowledge of the clinicopathologic parameters. The intensity of staining and the distribution of cytoplasmic positivity within the tumor cells were considered in the semi-quantitative assessment of the immunohistochemical results for both antibodies. The intensity of cytoplasmic staining was subjectively graded as 0 = low/absent; 1 = moderate; 2 = strong and distribution as 0: < 10%; 1: 10–50%; 2: > 50% for each protein. A case was considered negative when the total of the 2 scores was equal to 0, 1 or 2. A case was defined as positive when the total score was equal to 3 or 4.

Western blot

The correlations between WWP1 and ER/IGF-1R were analyzed in a panel of breast cancer cell lines by Western blot. Cells were cultured according to the ATCC recommended conditions and harvested using lysis buffer [50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 1% protease inhibitor cocktail I (Sigma, St. Louis, MO)]. The protein concentration was measured by using the D_C protein assay kit (Bio-Rad, Hercules,

CA). The proteins were subjected to SDS-PAGE gels and were transferred to a PVDF membrane. After blocking with 5% milk in PBST (PBS buffer with 0.1% Tween 20) for 1 hr at room temperature, the membrane was incubated with primary antibodies diluted with 3% BSA in PBST overnight at 4°C. The anti-ER α antibody (sc-7207) and the anti-IGF-1R β antibody (sc-713) were from Santa Cruz Biotechnology. The anti- β -actin mouse monoclonal antibody was from Sigma (A5441). The membrane was washed twice for 10 min, each with PBST and incubated with a secondary antibody diluted in 3% milk for 1 hr at room temperature. Following that, the membrane was washed 3 times and subjected to chemiluminescent substrate. Finally, images were documented by using the Fujifilm Imaging system LAS-3000.

WWP1 knockdown by siRNA

MCF7 and T47D cells were cultured in phenol red free DMEM media with 5% FBS, 0.01 mg/ml insulin, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate and 1% penicillin/streptomycin. MCF7 and T47D cells were transfected with 100 nM chemically synthesized Luc siRNA and WWP1 siRNA (Dharmacon, IL), respectively, by using lipofectamine 2000. The target sequences for WWP1 siRNA and the luciferase control siRNA were described in our previous study.³ Proteins were collected at 48 hr for Western blot. RNA was collected for RT-PCR. Primers and PCR conditions for ER have been described in previous studies.³²

DNA synthesis assays

For the DNA synthesis assay, MCF7 and T47D cells were seeded into 24-well plates ($\sim 1 \times 10^5$ /well) and transfected with WWP1 and control siRNA for 48 hr in media without serum and insulin as described above. The media were replaced with media containing 20 nM estradiol (E2, Sigma) for 2 days. Following that, the cells were labeled with ³H-thymidine (0.5 μ Ci/ml) for 4 hr. Upon the completion of treatment, the cells were washed with PBS once and fixed with 10% trichloroacetic acid. Radio-labeled DNA was solubilized by 100 μ l of 0.3 M NaOH and transferred to glass fiber filter membranes and radioactivity for ³H was measured using a Beckman-Coulter LS6500 multipurpose scintillation counter. For tamoxifen (Tam) treatment, MCF7 and T47D cells were cultured in normal media with 20 nM E2 and transfected with siRNA for 24 hr. After that, the media were replaced with media containing 20 nM E2 and 0–10 μ M Tam for 2 days. The DNA synthesis was measured as described above.

Statistical analysis

Statistical comparisons were carried out with the STATA software (Stata Corporation, College Station, TX). The χ^2 test was used to determine the significance of the associations between protein expression and clinicopathologic variables. Survival analysis was performed with univariate models and by the Kaplan-Meier method. The student *t*-test was performed to compare DNA synthesis. Dose-response curves in MCF7 and T47D were compared by 2-way ANOVA test (Sigmaplot 11.0). The level of significance was set at *p* = 0.05 or lower.

Results

Evaluation of the anti-WWP1 antibody for IHC

A mouse anti-WWP1 monoclonal antibody 1A7 (Novus Biologicals) was generated by using partial WWP1 recombinant protein (152–261 residues). This antibody was evaluated and then recommended for both Western blot and IHC by the manufacturer (<http://www.novusbio.com>).

We first evaluated the antibody 1A7 for Western blot by using our previous rabbit anti-human polyclonal anti-WWP1 antibody as a positive control.³ Both antibodies specifically recognize the exogenous WWP1 protein in HEK293T cells (data not shown). The en-

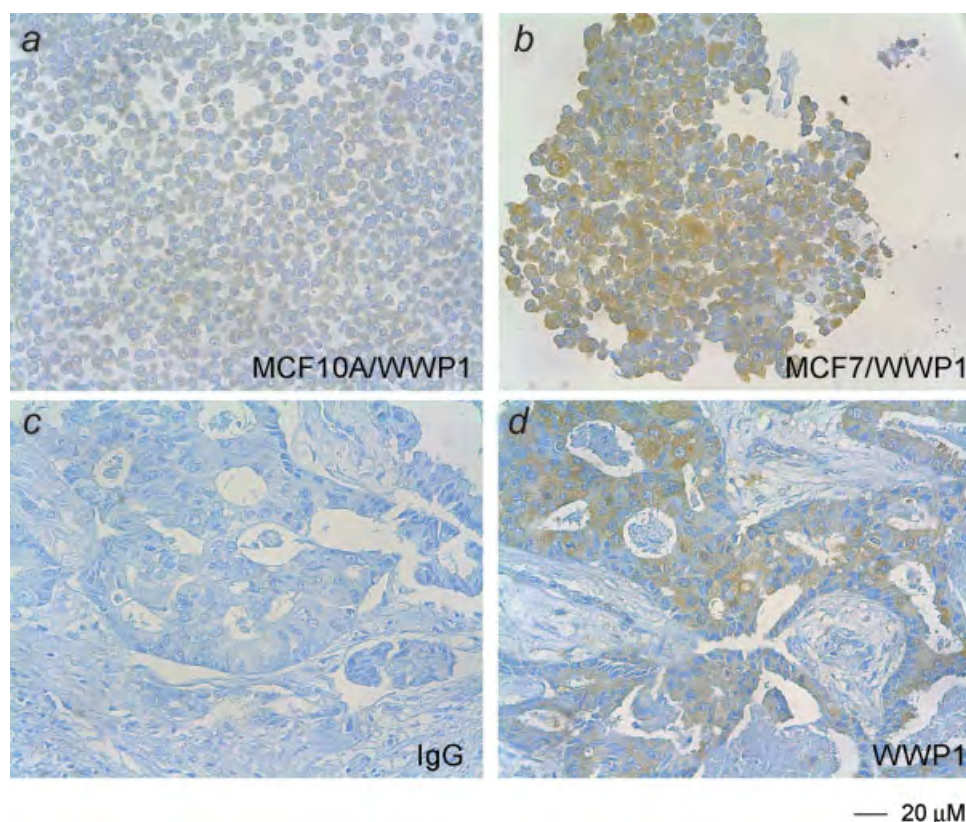


FIGURE 1 – Evaluation of the anti-WWP1 1A7 monoclonal antibody for IHC. (a) WWP1 staining in MCF10A cells (WWP1 is at a low level). (b) WWP1 staining in MCF7 cells (WWP1 is at a high level). (c) A breast tumor tissue stained by the IgG negative control. (d) The same breast tumor tissue in c stained by anti-WWP1 Ab. All WWP1 staining is in cytoplasm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ogenous WWP1 proteins in breast cancer cell lines can also be specifically detected by 1A7 (Figs. 4 and 5). These results indicate that 1A7 is a specific anti-WWP1 antibody for Western blot.

After that, we evaluated 1A7 for IHC by using MCF10A (WWP1^{low}) and MCF7 (WWP1^{high}) cell lines.⁴ Cytoplasmic staining was barely detected in MCF10A cells (Fig. 1a). The cytoplasmic signals were much stronger in MCF7 (Fig. 1b) under the same staining conditions. These results confirmed the previous Western blot results using a different anti-WWP1 antibody.⁴ To further test if 1A7 can be used for formalin-fixed, paraffin-embedded tissue sections, we stained a few invasive mammary carcinoma test cases (WWP1 statuses are unknown in these samples) and found that 1A7 generated moderate cytoplasmic staining in epithelial tumor cells but very low background staining in stromal cells in one testing slide (Fig. 1d). The control IgG did not show any staining in the same case under the same conditions (Fig. 1c). These data suggest that 1A7 could be used for WWP1 IHC in breast tumor tissues.

WWP1 expression by immunohistochemistry

We stained 187 cases of primary invasive mammary carcinoma. The WWP1 protein is barely detected in adjacent benign mammary gland epithelial cells (Fig. 2a) and normal breast epithelial cells (Fig. 3). In the same case 18, the WWP1 protein is strongly detected in invasive tumor cells (Fig. 2b). Although WWP1 has been previously reported to localize to the cytoplasmic membrane, cytoplasmic compartments and nucleus by immunofluorescence staining,^{17,33,34} we observed that WWP1 predominantly localizes to the cytoplasm by IHC. WWP1 is strongly overexpressed in some but not all invasive breast carcinomas. For example, Cases 119 and 160 (Figs. 2c and 2d) are positive but Cases 56 and 179 (Figs. 2e and 2f) are negative. In total, WWP1 is overexpressed in

40.6% (76/187) of the breast carcinoma clinical samples. In all positive cases, the WWP1 protein is predominantly localized to the cytoplasm of tumor cells. Cytoplasmic overexpression correlates with ER status as summarized in Table I ($p = 0.03$). However, no correlations between WWP1 and other clinicopathologic variables were identified.

The correlation between WWP1 and IGF-1R in breast tumors

Similar to WWP1, the IGF-1R protein was barely detected in normal breast epithelial cells (Fig. 3). Strong cytoplasmic expression of IGF-1R was observed in 110/183 (60.1%) breast tumors and correlated with the tumor subtype (64.4% IDC vs. 47.9% ILC, $p = 0.045$). Within the IDC subgroup, there is a trend that the expression of IGF-1R is correlated with ER ($p = 0.09$, not significant). There is a significant co-expression of both WWP1 and IGF-1R proteins ($p = 0.001$, Table II) in this cohort.

Consistent to the correlation among WWP1, ER and IGF-1R, triple positive and triple negative cases were identified easily. For example, Case 42 showed positive staining for cytoplasmic WWP1, cytoplasmic IGF-1R and nuclear ER (Fig. 3, the middle panel). In contrast, Case 9 showed negative for all 3 molecules. No correlation between WWP1 and HER2 was detected in this cohort.

The correlation of WWP1 with ER and IGF-1R in breast cancer cell lines

To further confirm the expression correlation among WWP1, ER and IGF-1R in breast tumors, we examined their expression in 12 breast cell lines by Western blot. Consistent with our previous report, the WWP1 protein was detected in normal human mammary epithelial cells (HMEC) and 2 immortalized mammary epi-

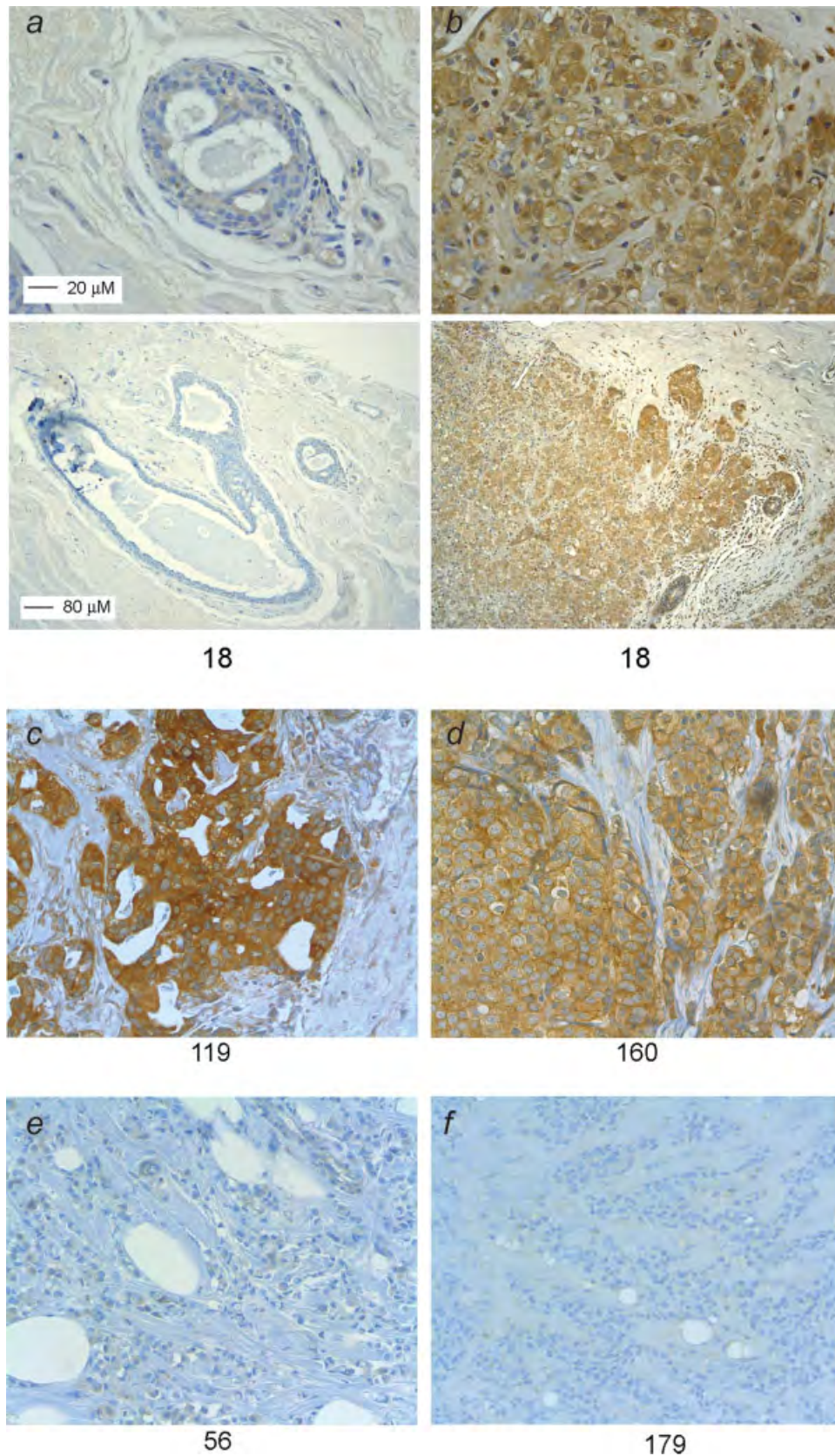


FIGURE 2 – Representative IHC results for WWP1 in breast carcinoma. (a) No WWP1 staining in benign breast epithelial cells in case 18 (69 year, T2N1M0, Grade II, ER+, PR+, HER2+, IGF-1R-). (b) Strong WWP1 staining in invasive breast tumor cells in Case 18. Pictures with different magnification (size bars are included) are shown for a,b. (c,d) Two breast tumors [Case 119 (50 year, T2N0M0, Grade III, ER+, PR+, HER2-, IGF-1R+) and 160 (65 year, T2N0M0, Grade III, ER+, PR-, HER2-, IGF-1R+)] show positive WWP1 staining. All WWP1 staining is in cytoplasm. (e,f) Two breast tumors [Case 56 (66 year, T2N1M0, grade I, ER-, PR-, HER2-, IGF-1R-) and 179 (44 year, T2N1M0, Grade II, ER+, PR+, HER2+, IGF-1R-)] show negative WWP1 staining.

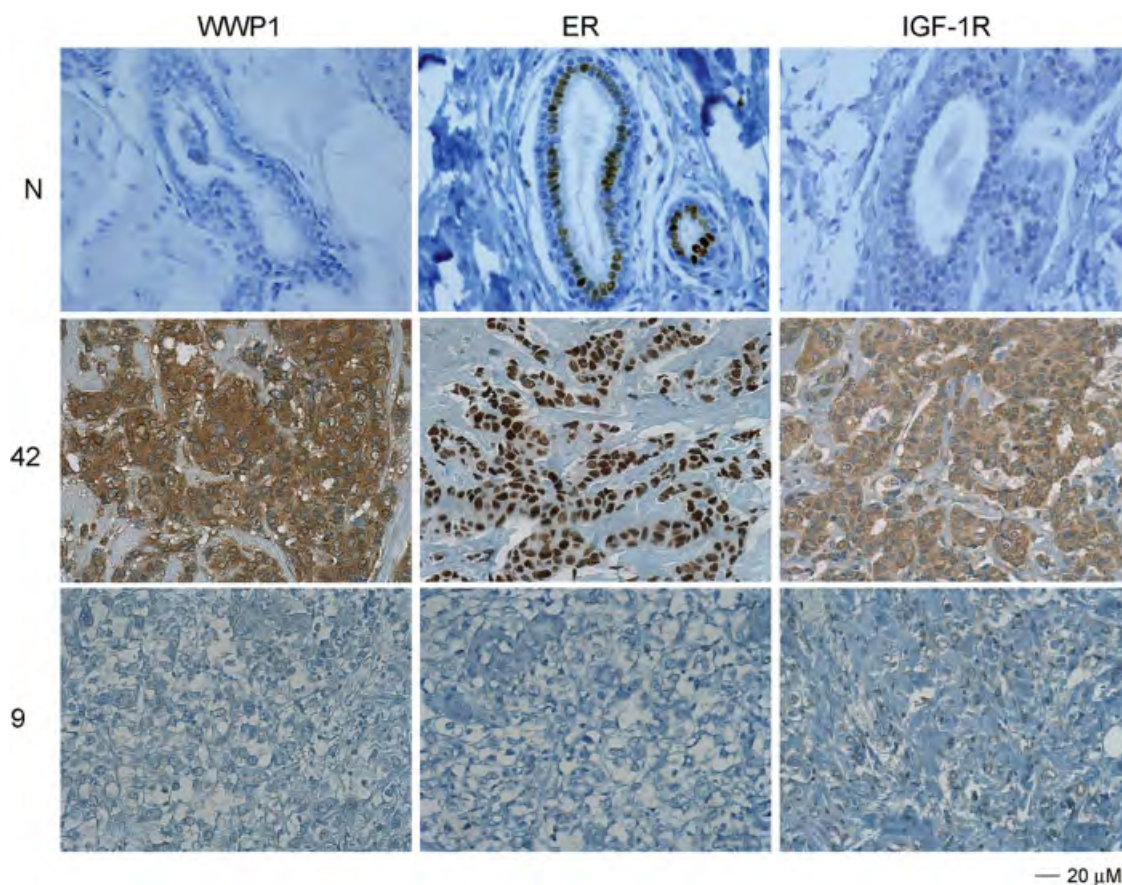


FIGURE 3 – Expression of WWP1, ER and IGF-1R in normal breast tissues and breast tumors. A normal (N) breast (49 year) shows negative WWP1, positive ER and very weak IGF-1R staining (Upper). Case 42 (68 year, T2N1M0, Grade III, PR+, HER2–) shows positive staining for WWP1, ER and IGF-1R (Middle). WWP1 and IGF-1R are in cytoplasm. ER is in nucleus. Case 9 (67 year, T1N1M0, Grade II, PR+, HER2–) shows negative staining for WWP1, ER and IGF-1R (lower).

TABLE I – CORRELATION BETWEEN WWP1 AND ER

	ER negative	ER positive	Total
WWP1 negative	47	57	104
WWP1 positive	22	53	75
Total	69	110	179

χ^2 test, $p = 0.031$.

TABLE II – CORRELATION BETWEEN WWP1 AND IGF-1R

	IGF-1R negative	IGF-1R positive	Total
WWP1 negative	52	52	104
WWP1 positive	20	56	76
Total	72	108	180

χ^2 test, $p = 0.001$.

thelial cell lines (MCF10A and 184B5) (Fig. 4). The ER levels are also low in these non-transformed cells. WWP1 is overexpressed in 4 ER positive breast cancer cell lines (MCF7, HCC1500, BT474 and MDA-MB-134) but not in 4 ER negative breast cancer cell lines (MDA-MB-231, BT20, MDA-MB-157, Hs578T). In T47D, both WWP1 and ER are in the middle levels. Interestingly, the expression pattern of IGF-1R fits very well with WWP1 and ER in these 12 breast cell lines (Fig. 4). Thus, the positive correlations between WWP1 and ER/IGF-1R were also observed in breast cell lines.

WWP1 maintains high ER levels

On the basis of the correlation between WWP1 and ER, we wondered if WWP1 is an estrogen responsive gene in breast cancer, or vice versa. To test this, we treated ER positive MCF7 cells with 20 nM E2 for different times but found no increase of the WWP1 protein level (data not shown). Then, we knocked down endogenous WWP1 by siRNA in MCF7 and T47D and found that the ER protein and mRNA levels are down-regulated (Figs. 5a and 5b). However, we found that the IGF-1R protein level is not

significantly affected by WWP1 knockdown (data not shown). Another WWP1 siRNA also generates similar results (data not shown).

WWP1 inhibition and tamoxifen collectively inhibits MCF7 and T47D cell proliferation

WWP1 is co-expressed with ER in breast cancer. Additionally, suppression of WWP1 down-regulates ER. Because ER is important for estrogen-stimulated breast cell growth and anti-estrogen treatment, we first wondered whether WWP1 inhibition decreases estrogen-stimulated cell proliferation. To this end, we knocked down WWP1 in MCF7 and T47D and treated cells with 20 nM E2. As measured by DNA synthesis (Fig. 5c), WWP1 knockdown significantly reduces cell proliferation in the absence and presence of E2 in both cell lines. The reduction is more dramatic in MCF7 than in T47D. However, we noticed that E2 still efficiently induces cell proliferation when WWP1 is knocked down by siRNA. These results imply that the rest of the ER proteins are sufficient to respond to estrogen in these 2 cell lines.

Following this, we decided to determine whether WWP1 knockdown will affect the effect of the anti-estrogen drug tamoxifen. As shown in Figure 5d, WWP1 knockdown and tamoxifen (in

a dosage dependent manner) significantly inhibits E2-mediated DNA synthesis in MCF7 and T47D ($p < 0.01$, 2 way ANOVA test). WWP1 knockdown and tamoxifen (1–10 μM) collectively suppresses cell proliferation in both T47D and MCF7. These results suggest that WWP1 inhibition may be combined with anti-estrogen therapy in the future.

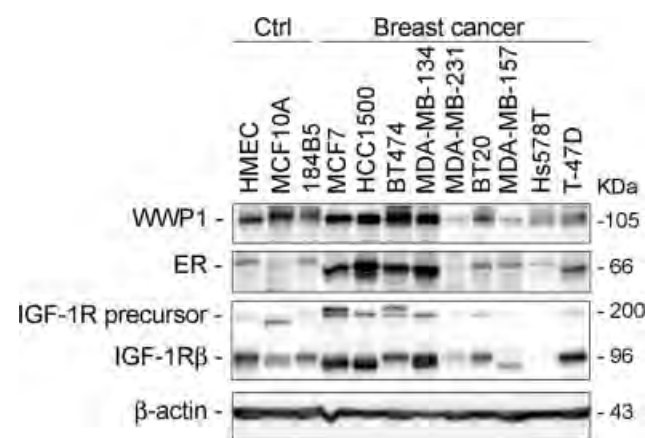


FIGURE 4 – The expression of WWP1, ER and IGF-1R proteins in a panel of breast cell lines. The protein levels were measured in 12 breast cell lines by Western blot. The WWP1 protein levels are higher in ER and IGF-1R positive breast cancer cell lines. The anti-IGF-1Rβ antibody detected both precursor (200 KDa) and IGF-1Rβ (96 KDa) bands. The variation of the IGF-1R protein size in different breast cell lines may be caused by different post-translational modification. β-actin was used for a loading control.

Discussion

In our previous report, WWP1 is frequently amplified and over-expressed in breast cancer and appears to associate with positive ER status.⁴ To further test whether WWP1 has the potential to become a useful clinical biomarker, we examined the WWP1 protein expression in 187 cases of primary invasive breast tumors by IHC. Although WWP1 is not associated with breast tumor stage, metastasis and survival, we confirmed that WWP1 is overexpressed in 40.6% of breast carcinoma and associated with ER. Interestingly, WWP1 is also associated with IGF-1R, but not with HER2 in this cohort.

While this study was ongoing, Nguyen Huu *et al.* reported that WWP1 could be used as a prognostic indicator for breast cancer by using a different anti-WWP1 antibody.²¹ In their cohort (354 primary breast tumors in a tissue microarray format), low/absent WWP1 protein expression is linked to a worse prognosis although no significant ($p = 0.14$) association between WWP1 and ER was observed. It is documented that positive ER²⁰ and IGF-1R^{25,27} are associated with non-invasive breast cancer in some studies, although we did not observe this in our cohort. Our results that WWP1 is lowly expressed in ER/IGF-1R negative breast

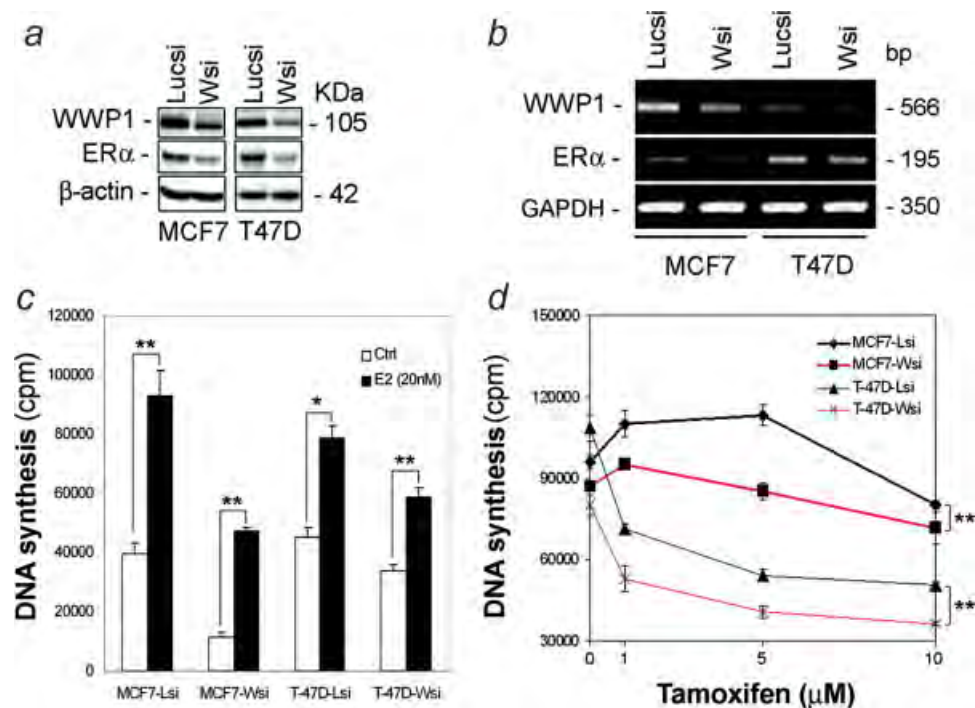


FIGURE 5 – WWP1 knockdown decreases the ER expression and suppresses cell proliferation alone or in combination with tamoxifen. (a) WWP1 knockdown decreases the ER protein levels in MCF7 and T47D. WWP1 is knocked down by an anti-WWP1 siRNA (Wsi). The anti-luciferase siRNA (Lucsi) was used as a negative control. All siRNA was transfected for 48 hr by using lipofectamine 2000 at 100 nM final concentration. (b) WWP1 knockdown decreases the ER mRNA levels in MCF7 and T47D, as determined by RT-PCR. (c) WWP1 siRNA significantly [$p < 0.05$ (t -test)] decreases basal cell proliferation in MCF7 and T47D. The cells were starved in serum/insulin/estrogen-free media for 2 days before adding E2. E2 significantly [$**p < 0.01$; $*p < 0.05$ (t -test)] promotes DNA synthesis in the absence and presence of WWP1 siRNA, $n = 3$. (d) WWP1 knockdown and tamoxifen collectively inhibit MCF7 and T47D cell growth. The cells were cultured in normal media and transfected with 100 nM siRNA for 1 day. Tamoxifen (1–10 μM) and E2 (20 nM) were added together to the siRNA transfected cells for 2 days. DNA synthesis was measured for cell proliferation. WWP1 knockdown and tamoxifen (1–10 μM) significantly [$**p < 0.01$ (Two way ANOVA test)] inhibits cell proliferation in MCF7 and T47D, $n = 3$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tumors and breast cancer cell lines (Figs. 3 and 4) are not against their conclusion that negative WWP1 is associated with poor prognosis. Using the monoclonal antibody, we did not observe any WWP1 nuclear staining that was reported by Nguyen Huu *et al.*,²¹ who used a rabbit polyclonal antibody for IHC. The different results may also be caused by different patients, patient numbers, tissue sizes and IHC protocols. Independent studies will be required to further validate these results in the future.

WWP1 may suppress breast cancer cell migration and metastasis because negative WWP1 is associated with negative ER (this study) and worse prognosis.²¹ Another WWP1 family member, Smurf2, has recently been shown to suppress breast cell metastasis in an E3 ligase activity dependent manner.³⁵ However, the mechanism of Smurf2 action and the expression of Smurf2 in breast cancer have not been studied. WWP1 is a strong inhibitor for the TGF β pathway that is well known to promote metastasis in the later stage of tumor development. Additionally, WWP1 has been shown to target several transcription factors, such as KLF5,¹⁷ RunX2¹⁴ and p63,¹⁹ for ubiquitin-mediated proteasomal degradation. KLF5 has been shown to promote migration.³⁶ RunX2 has long been associated with breast cancer metastasis by activating expression of bone matrix and adhesion proteins, matrix metalloproteinases and angiogenic factors.³⁷ Whether WWP1 truly suppresses breast cancer metastasis through inhibiting the TGF β pathway and transcription factors should be addressed in future studies.

Consistent with the expression association between WWP1 and ER, we found that WWP1 may positively regulate the ER transcription in breast cancer cells (Fig. 5). However, the exact mechanism by which WWP1 positively regulates the ER transcription is unclear at this time. Since WWP1 targets several transcription

factors for degradation, it is possible that WWP1 suppresses the negative transcriptional regulator of ER.

Additionally, WWP1 ablation significantly suppresses basal level cell proliferation and reduces E2-induced cell proliferation in MCF7 and T47D (Fig. 5c). However, the resting level of ER is sufficient for breast cancer cells to respond to E2. Nguyen Huu *et al.* reported that WWP1 is significantly associated with the mitotic index in clinical breast tumors,²¹ supporting our observation that WWP1 maintains breast cell proliferation. Recently, we discovered that WWP1 also upregulates ErbB2/HER2 in MCF7.¹³ It is possible that WWP1 maintains cell proliferation not solely through ER.

Our previous study also suggested that WWP1 ablation induces cell growth arrest and apoptosis in ER positive breast cancer cell lines MCF7 and HCC1500.⁴ In this study, we further tested whether WWP1 knockdown and tamoxifen can be combined to collectively suppress ER positive breast cancer cell proliferation. Our results suggest that WWP1 inhibition can reduce MCF7 and T47D cell growth in combination with tamoxifen. In line with our observations, WWP1 knockdown has recently been shown to suppress anchorage-independent growth in these 2 cell lines.²¹ These results further support that WWP1 inhibition may be a novel promising approach for ER positive breast cancer patients.

In summary, WWP1 is overexpressed in breast tumor cells when compared with normal and adjacent benign epithelial cells and is associated with the ER/IGF-1R phenotype in both breast cancer cell lines and breast carcinoma clinical samples. Using WWP1 knockdown strategies, targeting WWP1 decreases ER expression levels and suppresses cell growth alone or in combination with tamoxifen in ER positive breast cancer cell lines. Further development of WWP1 as a biomarker and therapeutic target for ER positive breast cancer management deserves further investigation.

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ORIGINAL ARTICLE

WW domain containing E3 ubiquitin protein ligase 1 targets the full-length ErbB4 for ubiquitin-mediated degradation in breast cancer

Y Li¹, Z Zhou¹, M Alimandi² and C Chen¹

¹The Center for Cell Biology and Cancer Research, MS355, Albany Medical College, Albany, NY, USA and ²Department of Experimental Medicine and Pathology, University 'La Sapienza', Rome, Italy

ErbB4, a member of the epidermal growth factor receptor family, plays a role in normal breast and breast cancer development by regulating mammary epithelial cell proliferation, survival and differentiation. In this study, we show that WWP1, a C2-WW-HECT type E3 ubiquitin ligase, binds, ubiquitinates and destructs ErbB4-CYT1, but much less efficiently for CYT2, isoforms (both JMa and JMb). The protein–protein interaction occurs primarily between the first and third WW domains of WWP1 and the second PY motif of ErbB4. Knockdown of WWP1 by two different small interfering RNAs increases the endogenous ErbB4 protein levels in both MCF7 and T47D breast cancer cell lines. In addition, overexpression of the wild type, but not the catalytic inactive WWP1, dramatically decreases the endogenous ErbB4 protein levels in MCF7. Importantly, we found that WWP1 negatively regulates the heregulin- β 1-stimulated ErbB4 activity as measured by the serum response element report assay and the BRCA1 mRNA expression. After a systematic screening of all WWP1 family members by small interfering RNA, we found that AIP4/Itch and HECW1/NEDL1 also negatively regulate the ErbB4 protein expression in T47D. Interestingly, the protein expression levels of both WWP1 and ErbB4 are higher in estrogen receptor- α -positive than in estrogen receptor- α -negative breast cancer cell lines. These data suggest that WWP1 and its family members suppress the ErbB4 expression and function in breast cancer.

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Introduction

The WW domain containing E3 ubiquitin protein ligase 1 (WWP1) belongs to the C2-WW-HECT type E3 family, which comprises of nine members including

AIP4/Itch and WWP1 (Chen and Matesic, 2007). The N-terminal C2 domain is responsible for calcium-dependent phospholipid binding. The middle four WW domains recognize substrates with PY (PPXY) motifs. The C-terminal HECT domain is the enzyme catalytic center for the ubiquitin transfer. WWP1 is over-expressed in prostate cancer (Chen *et al.*, 2007a) and estrogen receptor- α (ER- α)-positive breast cancers and promotes breast cell proliferation and survival (Chen *et al.*, 2007b, 2009; Nguyen Huu *et al.*, 2008). WWP1 is an intrinsic E3 ubiquitin ligase for multiple PY motifs containing transcription factors, including Smad2 (Seo *et al.*, 2004), RunX2 (Jones *et al.*, 2006), KLF5 (Chen *et al.*, 2005) and p63 (Li *et al.*, 2008b). In addition, WWP1 may indirectly regulate several receptors, including ER- α (Chen *et al.*, 2009), epidermal growth factor receptor (EGFR) and ErbB2 (Chen *et al.*, 2008).

The EGFR subfamily of receptor tyrosine kinases consists of four homologous members: EGFR, ErbB2, ErbB3 and ErbB4 (HER4). Unlike other ErbB family members, ErbB4 may have tumor-suppressor activities because ErbB4 decreases breast epithelial cell proliferation (Pitfield *et al.*, 2006; Muraoka-Cook *et al.*, 2006a; Feng *et al.*, 2007), and promotes differentiation (Muraoka-Cook *et al.*, 2006b, 2008) and apoptosis (Naresh *et al.*, 2006; Vidal *et al.*, 2007). Consistently, many studies suggest that the ErbB4 expression correlates with a good prognosis in breast cancer (Kew *et al.*, 2000; Witton *et al.*, 2003; Barnes *et al.*, 2005; Koutras *et al.*, 2008). However, inconsistent results have also been frequently reported (Tang *et al.*, 1999; Junttila *et al.*, 2005; Maatta *et al.*, 2006; Zhu *et al.*, 2006). Nevertheless, ErbB4 seems not to be a strong tumor suppressor because ErbB4 knockout does not affect ErbB2-induced breast tumorigenesis (Jackson-Fisher *et al.*, 2006).

ErbB4 is a 180-kDa glycoprotein consisting of a ligand-binding extracellular region, a hydrophobic transmembrane region, and a cytoplasmic region including a tyrosine kinase domain and a cytoplasmic tail (Figure 1a). Alternative splicing of the ErbB4 transcripts generates four mRNA variants that differ in the sequence encoding the extracellular juxtamembrane region (JMa and JMb) and the cytoplasmic region (CYT-1 and CYT-2). The JMa isoforms, unlike the JMb isoforms, include a cleavage site for the tumor necrosis factor- α -converting enzyme (TACE) on ligand binding. ErbB4 first cleaved by TACE is a substrate for γ -secretase that releases a soluble 80-kDa intracellular

Correspondence: Dr C Chen, The Center for Cell Biology and Cancer Research, Albany Medical College, MS355, Mail code 165, 47 New Scotland Ave., Albany, NY 12208, USA.
E-mail: chenc@mail.amc.edu

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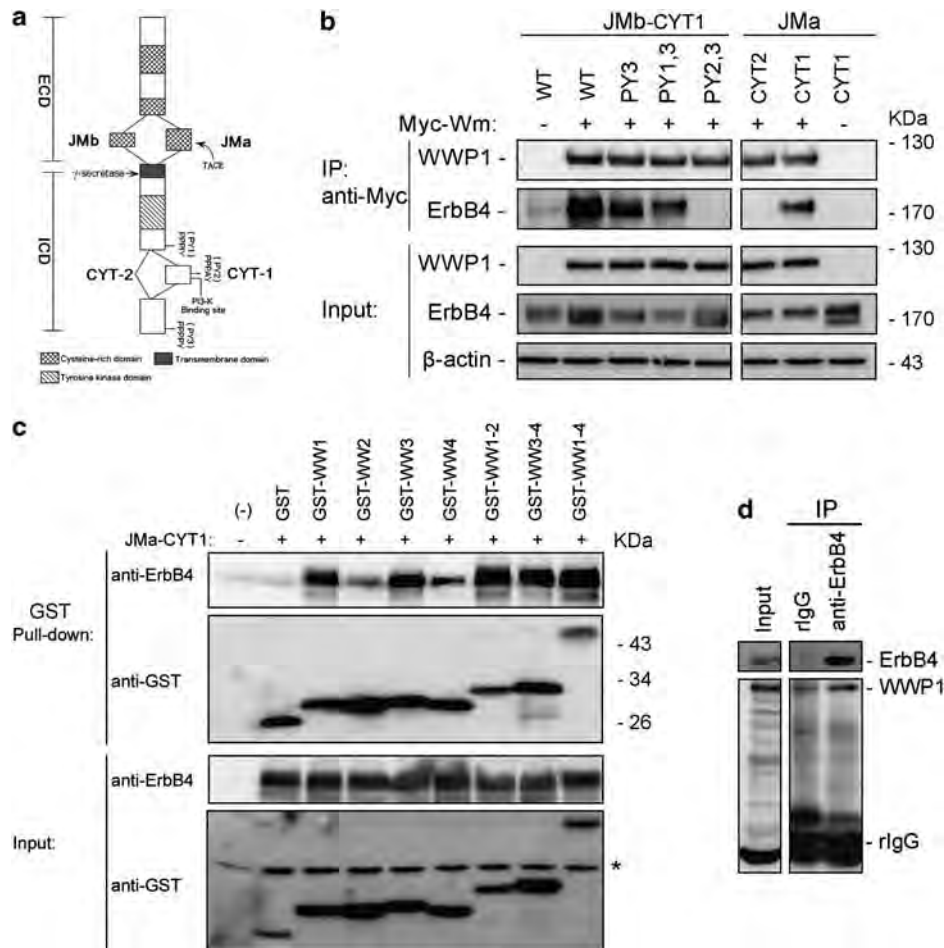


Figure 1 The WWP1 protein interacts with ErbB4 proteins through the WW/PY motifs in mammalian cells. **(a)** Schematic diagram of ErbB4 isoforms. Alternative splicing generates four ErbB4 isoforms because of a difference at the juxtamembrane (JM) or cytoplasmic (CYT) domains. JMa isoforms, unlike JMb isoforms, include a cleavage site for tumor necrosis factor- α -converting enzyme (TACE), and can be cleaved by TACE, resulting in the shedding of the extracellular domain (ECD), which is a substrate of γ -secretase that releases a soluble intracellular domain (ICD) from the cell membrane. CYT-1 isoforms, unlike CYT-2 isoforms, contain a 16-amino acid sequence that includes a PI3-K binding site (YTPM) as well as a proline-rich protein interaction motif (PPAY). **(b)** Both JMb-CYT1 and JMa-CYT1 are co-immunoprecipitated with Myc-WWP1C886S (Wm), and PY3 and PY1, 3 mutants also interact with WWP1. However, the PY2, 3 mutant of JMb-CYT1 and JMa-CYT2 (having no second PY motif isoform) cannot be co-immunoprecipitated with Myc-Wm. HEK293T cells were co-transfected with different combinations of expression plasmids for Myc-Wm, JMb-CYT1 wild type (WT), PY3, PY1, 3, PY2, 3, JMa-CYT1 and JMa-CYT2. immunoprecipitation (IP) was carried out using anti-Myc antibody (Ab). Myc-Wm was probed by anti-WWP1 Ab. The catalytic inactive WWP1 mutant was used to avoid ErbB4 degradation by WT WWP1. β -actin serves as a loading control for the input. **(c)** WWP1 binds to ErbB4-JMa-CYT1 through the first and third WW domains, as determined by glutathione S-transferase (GST) pull-down assays. Four WW domains of WWP1 were individually or collectively expressed as GST fusion proteins in the HEK293T cells. **(d)** The endogenous WWP1 protein forms a complex with the endogenous ErbB4 (JMa-CYT1) protein in MCF7. The ErbB4 protein was probed with the rabbit anti-ErbB4 Ab. The same amount of rabbit IgG nonspecifically immunoprecipitated few WWP1 after extensive washing. However, anti-ErbB4 Ab immunoprecipitated much more WWP1 than the IgG control under the same conditions.

domain that can translocate into the nucleus and regulate gene transcription. The ErbB4-CYT1 isoforms contain a 16-amino acid peptide with a PY motif that can be recognized by WW domains (Omerovic *et al.*, 2004). There are two additional PY motifs in ErbB4-CYT1 isoforms (Figure 1a). Several WW domains containing proteins, including WWOX (Aqeilan *et al.*, 2005), YAP (Komuro *et al.*, 2003; Omerovic *et al.*, 2004) and AIP4/Itch (Omerovic *et al.*, 2007), have been reported to bind to ErbB4 through these PY motifs.

As both WWP1 and ErbB4 play important roles in breast cancer, we hypothesize that WWP1 regulates the ErbB4 activity through the WW/PY motif interaction. Here, we show that WWP1 specifically interacts with and targets the ErbB4-CYT1 isoforms for ubiquitin-mediated degradation and modulates the ligand-dependent ErbB4 activity. These findings shed light on the mechanism of WWP1 action and may help design future breast cancer target therapy.

Results

WWP1 interacts with ErbB4 mainly through the first and third WW domains of WWP1 and the second PY motif of ErbB4

The ErbB4 protein has been shown to interact with several WW domains containing proteins, such as Itch, WWOX and YAP, through the WW/PY motif interaction (Aqeilan *et al.*, 2005; Omerovic *et al.*, 2007). To test whether WWP1 also interacts with ErbB4, we first carried out the co-immunoprecipitation (IP) experiments. A plasmid expressing Myc-WWP1C886S (a catalytic inactive mouse WWP1 mutant) and plasmids expressing ErbB4 (different isoforms) were transfected into HEK293T cells. Myc-WWP1C886S was efficiently immunoprecipitated by the anti-Myc antibody (Ab) (Figure 1b). We found that both JMa-CYT1 and JMb-CYT1 isoforms are co-immunoprecipitated with Myc-WWP1C886S. The anti-Myc Ab itself cannot immunoprecipitate the ErbB4 proteins without the expression of Myc-WWP1C886S, suggesting that the protein–protein interactions are specific.

Then we tested whether the protein–protein interaction between WWP1 and ErbB4 is through the WW/PY motifs and which PY motif is involved in the interaction. We mutated the PY motifs in ErbB4-JMb CYT1 (PY1, PY1, 3 and PY2, 3) by substituting tyrosine (Y) with phenylalanine (F). We found that ErbB4-JMb CYT1-PY3 and -PY1, 3 mutants still interact with Myc-WWP1C886S; however, the ErbB4-JMb CYT1-PY2, 3 mutants almost completely loses the interaction with Myc-WWP1C886S (Figure 1b). A similar result was observed for the ErbB4-JMa isoform. The ErbB4-JMa-CYT2, which does not have the second PY motif, almost never interacts with Myc-WWP1C886S at all (Figure 1b). These results suggest that the second PY motif in ErbB4-CYT1 is essential for WWP1 interaction and the CYT2 isoforms do not interact with WWP1 efficiently in spite of the existence of two other PY motifs.

Then, we asked which WW domain of WWP1 participates in the protein interaction with ErbB4. We fused each of the four WW domains of WWP1 to the C-terminus of glutathione S-transferase (GST) and carried out GST pull-down assays with ErbB4-JMa-CYT1 in HEK293T cells. As shown in Figure 1c, GST itself does not pull down any ErbB4. All four GST-WW proteins pull down different amounts of ErbB4. GST-WW1 and GST-WW3 pull down a similar and large amount of ErbB4, whereas GST-WW2 and GST-WW4 pull down much less ErbB4, although the expression levels of GST-WW and ErbB4 are similar in the four different groups. Consistently, GST-WW (1–2), GST-WW (3–4) and GST-WW (1–4) efficiently pull down ErbB4. It seems that more WW domains bind to more ErbB4. These findings suggest that the first and third WW domains play major roles for ErbB4 binding and the second and fourth WW domains also contribute to recognizing ErbB4. Taken together, the protein interaction between WWP1 and ErbB4 is primarily through the first and third WW domains of WWP1.

Finally, we examined whether the endogenous WWP1 protein interacts with the endogenous ErbB4 protein. The ErbB4 (JMa-CYT1 (Maatta *et al.*, 2006)) proteins were immunoprecipitated from MCF7 by using the anti-ErbB4 Ab, and the endogenous WWP1 protein was detected in the same complex (Figure 1d). This result suggests that the protein–protein interaction between WWP1 and ErbB4 occurs at the physiological level.

WWP1 ubiquitinates ErbB4 in cultured mammalian cells

As the WWP1 E3 ligase interacts with both ErbB4-CYT1 isoforms, we asked whether WWP1 ubiquitinates the ErbB4-CYT1 proteins in mammalian cells. To this end, we transfected the expression constructs for wild-type (WT) human WWP1, the catalytic inactive hWWP1C890A mutant, ErbB4, and Myc-Ub into HEK293T cells. We carried out IP with the anti-ErbB4 Ab together with protein A/G agarose beads under a denaturing condition to eliminate any ErbB4-associated proteins through non-covalent bonds. The ubiquitin-conjugated ErbB4 proteins were detected by western blot with anti-Myc Ab. As shown in Figure 2a, WT, but not the catalytic inactive WWP1, significantly increases the ubiquitination of ErbB4-CYT1 (both JMa and JMb) compared with the vector control. The format of ErbB4 ubiquitination by WWP1 is most likely polyubiquitination because a smear of bands above the unmodified ErbB4 was detected. These results indicate that WWP1 polyubiquitinates ErbB4-CYT1 isoforms through its E3 ligase activity.

As WWP1 does not interact with ErbB4 isoforms without the second PY motif, we examined the ubiquitination of the ErbB4-JMb-CYT1-PY3, -PY1, 3 and -PY2, 3 and -JMa-CYT2 by WWP1. Consistent with the protein interaction results, WWP1 less efficiently ubiquitinates ErbB4-JMb-CYT1-PY3 and -PY1, 3 isoforms but barely ubiquitinates the ErbB4-JMb-CYT1-PY2, 3 and ErbB4-JMa-CYT2 isoforms under the same conditions. We conclude that the protein interaction is essential for WWP1 to ubiquitinate ErbB4.

To investigate whether endogenous WWP1 contributes to the endogenous ErbB4 ubiquitination, we knocked down WWP1 in T47D by an anti-WWP1 small interfering RNA (siRNA) and examined the ubiquitination of ErbB4 (JMa-CYT1 (Maatta *et al.*, 2006)). Compared with the Luc siRNA, the anti-WWP1 siRNA efficiently silences the WWP1 protein expression and decreases the ubiquitinated ErbB4 (Figure 2b).

WWP1 promotes ErbB4 protein degradation

To test whether WWP1 promotes ErbB4 protein degradation, we first measured the steady-state levels of ErbB4-CYT1 in the presence and absence of WWP1. As shown in Figure 3a, the steady-state levels of ErbB4-CYT1 (both JMa and JMb isoforms) are dramatically decreased in WT WWP1 but not in the WWP1C890A overexpressing HEK293T cells. As expected, WT WWP1 fails to dramatically decrease the steady-state level of ErbB4-JMa-CYT2 (Figure 3a) compared with WWP1C890A because JMa-CYT2 cannot interact with

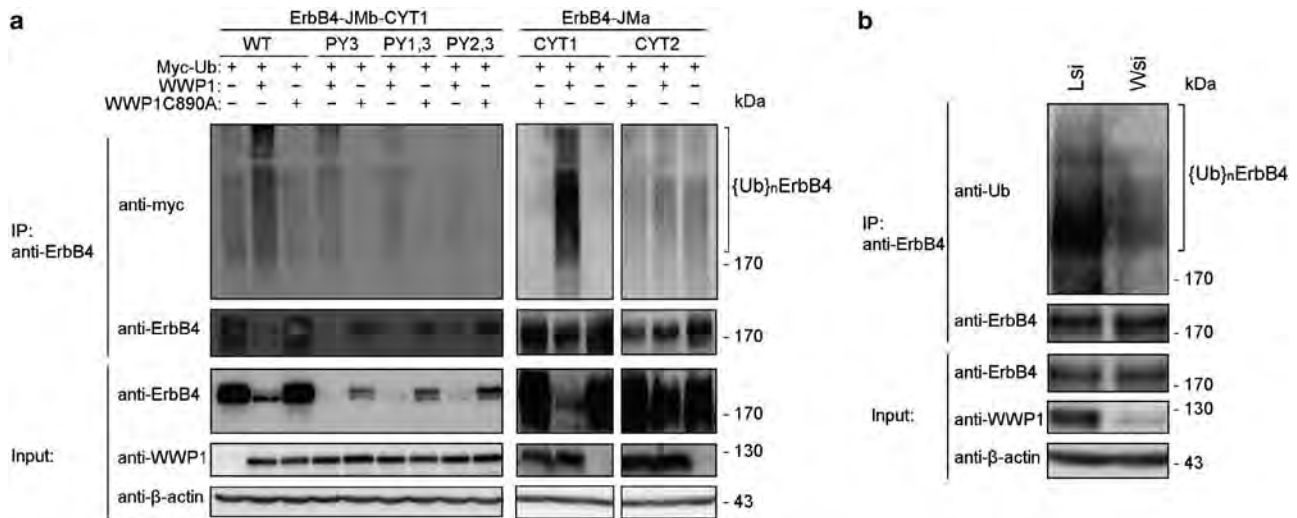


Figure 2 WWP1 ubiquitinates ErbB4 proteins in mammalian cells. **(a)** WWP1 ubiquitinates both juxtamembrane (JM)a-cytoplasmic (CYT)1 and JMb-CYT1 isoforms using its E3 ligase activity. WWP1 also ubiquitinates the PY3 and PY1, 3 mutants of JMb-CYT1 with reduced efficiency, but it cannot efficiently ubiquitinate the PY2, 3 mutant of JMb-CYT1 and JMa-CYT2 isoforms. HEK293T cells were co-transfected with expressing plasmids for Myc-Ub, WWP1, WWP1C890A, JMa-CYT1, JMa-CYT2 and JMb-CYT1 (wild type (WT), PY3, PY1, 3 and PY2, 3 mutants) as indicated. The cells were treated with 20 μM of the proteasome inhibitor, MG132, overnight to accumulate the ubiquitinated ErbB4 species. The immunoprecipitation (IP) was carried out with the anti-ErbB4 antibody (Ab) with protein A/G beads under denaturing conditions. Immunoblotting was carried out with the indicated Abs. The ubiquitin-conjugated ErbB4 proteins were detected by anti-Myc Ab. **(b)** The endogenous WWP1 E3 ligase ubiquitinates the endogenous ErbB4 protein in T47D. WWP1 was knocked down by anti-WWP1 siRNA#1 (Wsi). The anti-luciferase small interfering RNA (siRNA) (Lsi) was used as the negative control. MG132 was added to the cells for overnight. IP was carried out under a denaturing condition using anti-ErbB4 Ab. The ubiquitin-conjugated ErbB4 proteins were detected by anti-Ub Ab.

WWP1 nor be ubiquitinated by WWP1. In addition, we found that WT WWP1, but not WWP1C890A, decreases the steady-state levels of ErbB4-JMa-CYT1 in a dosage-dependent manner (Figure 3b). We did observe that a high level of WWP1 or WWP1C890A can slightly decrease the ErbB4-JMa-CYT2 protein levels by an unknown reason (Figures 3a and b).

To further investigate whether the decrease of ErbB4 by WWP1 is because of the increase of protein degradation, we measured the half-lives of ErbB4 isoforms in the presence and absence of WWP1 by cycloheximide chase assays. As shown in Figures 3c and d, all tested ErbB4 isoforms (CYT1 and CYT2) show a long half-life (>10 h) in HEK293T cells. When WT WWP1 is co-expressed, the half-lives of ErbB4-CYT1 isoforms are dramatically decreased to about 2 h (Figures 3c and d). The catalytic inactive WWP1C890A does not significantly affect the half-life of ErbB4-CYT1 isoforms. Consistent with the results that WWP1 ubiquitinates the ErbB4-JMb-CYT1-PY3 and -PY1, 3 isoforms with reduced efficiencies, WWP1 decreases the half-lives of these mutants less efficiently (~3.5 h for PY3 and ~4 h for PY1, 3). In addition, ErbB4-JMb-CYT1-PY2, 3 and ErbB4-JMa-CYT2 isoforms are resistant to WWP1-mediated degradation (Figures 3c and d) because of the lack of protein ubiquitination by WWP1.

To further characterize the mechanism by which ErbB4 is degraded after ubiquitination by WWP1, we carried out cycloheximide chase assays in the presence of the proteasome inhibitor, MG132, and the lysosome

inhibitor, chloroquine. As shown in Figures 3c and d, WWP1-induced ErbB4 degradation is partly blocked by MG132, but not chloroquine, suggesting that the degradation of ErbB4 by WWP1 is partly through the 26S proteasome, not by the lysosome.

WWP1 downregulates the endogenous ErbB4 protein levels in breast cancer cells

To determine whether WWP1 targets ErbB4 for degradation under physiological conditions, we knocked down endogenous WWP1 by two different anti-WWP1 siRNAs in two breast cancer cell lines, T47D and MCF7, expressing ErbB4-JMa-CYT1 (Maatta *et al.*, 2006). We found that the endogenous ErbB4 protein levels are remarkably elevated in both cell lines when WWP1 is knocked down by both siRNAs (Figure 4a). The ErbB4 mRNA levels are not changed by WWP1 knockdown in these cells (data not shown). These results suggest that WWP1 targets the endogenous ErbB4 protein for degradation in breast cancer cell lines.

In addition, we stably overexpressed WWP1 and WWP1C890A in MCF7. The expression of WT and mutant WWP1 in MCF7 populations was shown by western blotting (Figure 4b). We found that WT WWP1 dramatically decreases the endogenous ErbB4 protein level compared with LacZ and WWP1C890A (Figure 4b). For an unknown reason, WWP1C890A also decreases the ErbB4 protein level compared with LacZ. To exclude the possibility that the regulation is at the mRNA level, we examined the ErbB4 mRNA levels

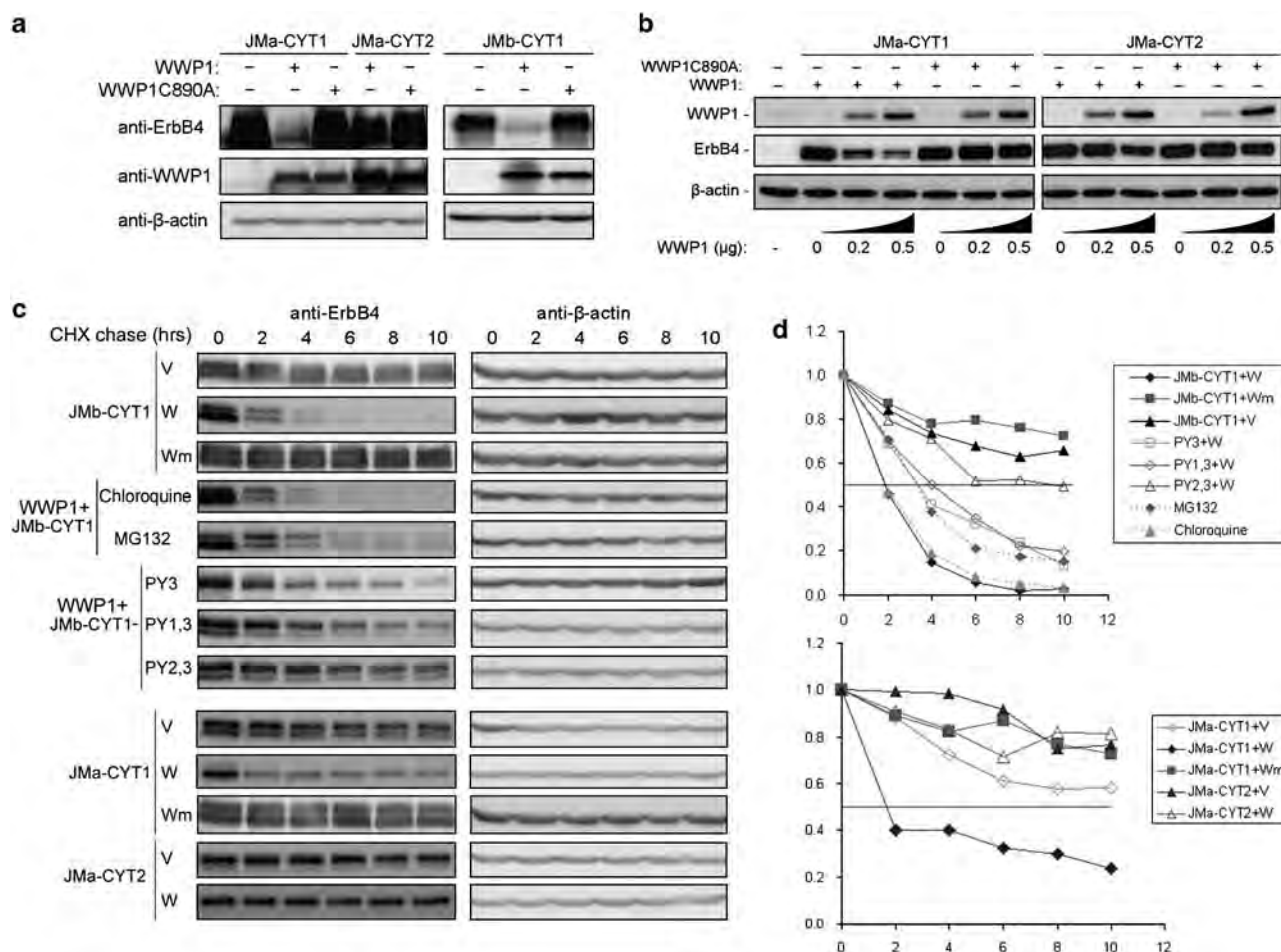


Figure 3 WWP1 promotes ErbB4 protein degradation. (a) Wild-type (WT) WWP1, but not the catalytic inactive mutant, WWP1C890A, decreases the steady protein levels of juxtamembrane (JM)a-cytoplasmic (CYT)1 and JMb-CYT1, but not JMa-CYT2, in HEK293T cells as determined by western blot. An empty vector was used as the negative control. (b) WT WWP1, but not WWP1C890A, decreases the steady protein levels of JMa-CYT1, but not JMa-CYT2, in a dosage-dependent manner (0, 0.2 and 0.5 μg WWP1-expressing plasmids). (c) Measurement of protein half-lives by cycloheximide (CHX) chase assays. HEK293T cells were co-transfected with the indicated plasmids. At 48 h after transfection, the cells were incubated with 50 μg/ml CHX for different times (2–10 h) and were collected for western blot. MG132 (20 μM) and chloroquine (100 μM) were added together with CHX for the indicated groups. β-actin was used as a control. The exposure times for each panel have been adjusted to better compare protein half-lives. (d) Quantitative results of panel c by the IMAGE J software. The β-actin normalized ErbB4 protein levels at 0 h were defined as 1.0 for each panel. V, empty vector; W, WWP1; Wm, WWP1C890A.

Figure 4 WWP1 regulates the endogenous ErbB4 protein levels and activities in breast cancer cell lines. (a) Knockdown of WWP1 by two different small interfering RNAs (siRNAs) increases the endogenous ErbB4 (JMa-CYT1) protein levels in the T47D and MCF7 breast cancer cell lines, as determined by western blot. Luciferase (L) siRNA was used as the negative control. Mock means no siRNA. All siRNAs were transfected at 100 nM final concentration for 48 h. (b) Wild-type (WT) WWP1, but not WWP1C890A, overexpression dramatically decreases the endogenous ErbB4 protein expression in MCF7. WT and mutant WWP1C890A were transduced into MCF7 by a lentiviral system. LacZ was used as the negative control. The blasticidin (10 μg/ml)-resistant cell populations were used for western blot. The WWP1C890A mutant also slightly decreases the ErbB4 levels in MCF7 for an unknown reason. (c) Measurement of endogenous ErbB4 half-lives by cycloheximide (CHX) chase assays. WWP1 was knocked down in T47D by anti-WWP1 siRNA #1 (Wsi) and the control siRNA (Lsi) for 2 days. The cells were incubated with 50 μg/ml CHX for different times (2–4 h) and were harvested for western blotting. β-actin was used as a loading control. (d) Quantitative results of ErbB4 degradation from panel c by IMAGE J software. (e) MCF7 cells were transfected with the serum response element (SRE)-Luc plasmid together with luciferase (L), WWP1 (W) and ErbB4 (E) siRNAs. Twenty-four hours later, the transfected cells were serum-starved overnight and were stimulated with heregulin (HRG)-β-1 (100 ng/ml) for 8 h. Cell lysates from three parallel wells were pooled, and equal amounts of proteins were subjected to immunoblotting for ErbB4 and WWP1. (f) WWP1 regulates the SRE promoter activity through ErbB4. The average relative luciferase activities are plotted. WWP1 siRNA significantly increases the HRG-β-1-induced SRE promoter activity in MCF7, whereas ErbB4 siRNA significantly decreases the HRG-β-1-induced SRE promoter activity in MCF7. Knockdown of ErbB4 can partially rescue WWP1 siRNA-induced SRE promoter activity. All statistics were analysed by a *t*-test. ***P* < 0.01. (g) Knockdown of ErbB4 can partially rescue WWP1 siRNA-induced BRCA1 mRNA expression in the presence of HRG-β-1 (100 ng/ml) in MCF7. The normalized BRCA1 mRNA levels were labeled below each lane. Overexpression of WT WWP1 and WWP1C890A decreases BRCA1 mRNA levels in the presence of HRG-β-1 (100 ng/ml) in MCF7.

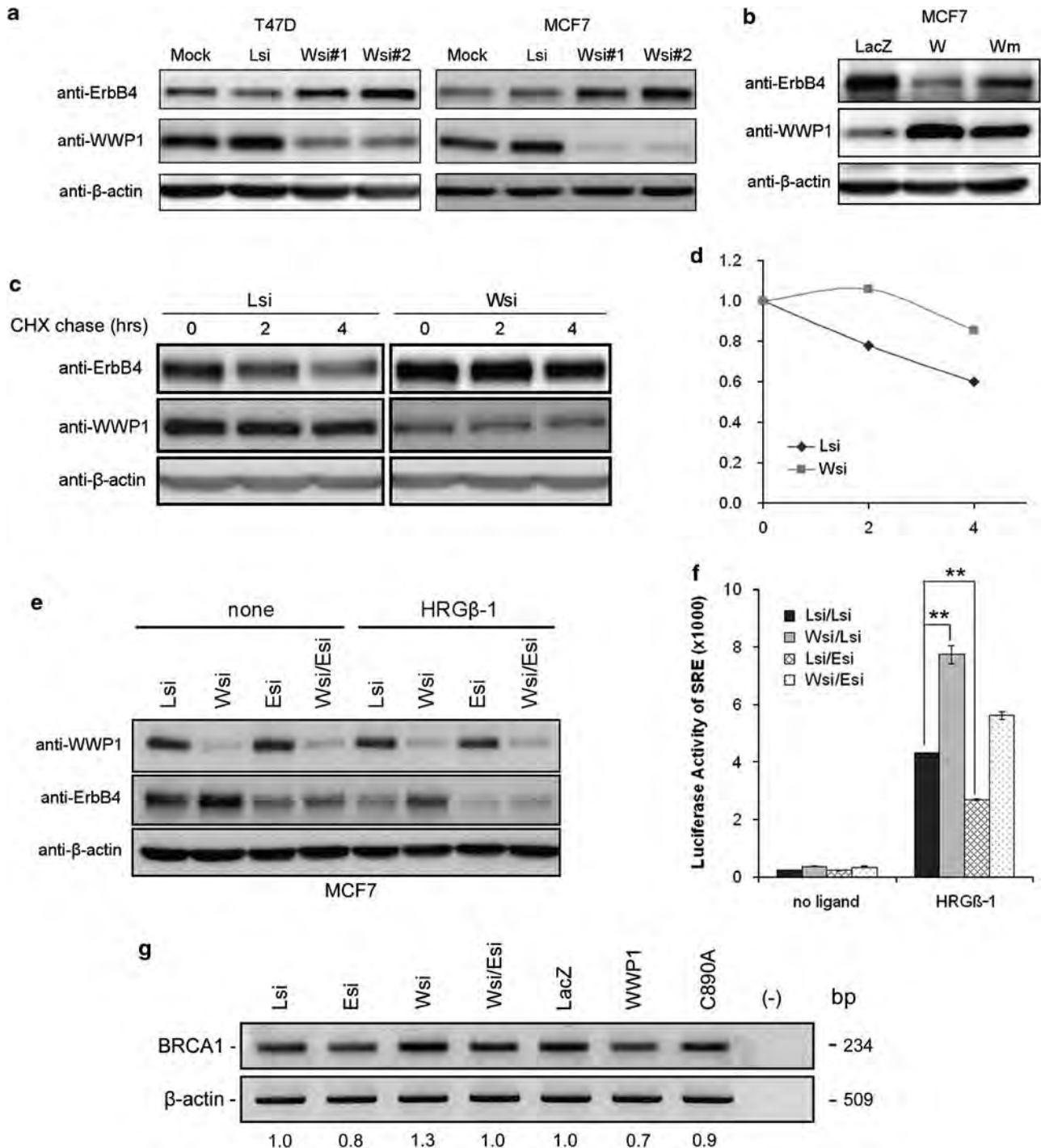
when WWP1 is overexpressed in MCF7 by reverse transcriptase-PCR and found that the ErbB4 mRNA levels are not significantly affected by WWP1 (data not shown). Taken together, we conclude that WWP1 regulates the endogenous ErbB4 protein expression in breast cancer cell lines.

To further test whether the endogenous WWP1 protein targets the endogenous ErbB4 protein for degradation, we knocked down WWP1 in T47D by

the anti-WWP1 siRNA and examined the half-lives of ErbB4. Compared with the Luc siRNA, the WWP1 siRNA indeed dramatically increases the half-life of ErbB4 (Figures 4c and d).

WWP1 inhibits the ErbB4 biological activities in breast cancer

As a receptor tyrosine kinase, ErbB4 initiates signaling on binding to ligands such as heparin-binding EGF-like



growth factor, betacellulin, epiregulin and heregulin (HRG) (Sundvall *et al.*, 2008). It has been reported that HRG can activate the serum response element (SRE) through ErbB4 (Omerovic *et al.*, 2007). To investigate whether WWP1 inhibits the ErbB4 downstream signaling, we measured the ErbB4 activities using the SRE luciferase reporter assay after knocking down WWP1 and ErbB4 in MCF7. The knockdown effect of WWP1 and ErbB4 is shown in Figure 4e. As expected, knockdown of WWP1 increases the ErbB4 expression levels in the absence and presence of HRG- β -1. In contrast, knockdown of ErbB4 has no effect on the WWP1 expression. Another anti-ErbB4 siRNA has the same result (data not shown). As shown in Figure 4f, the ErbB4 activity is dramatically increased after HRG- β -1 stimulation. WWP1 knockdown significantly increases but ErbB4 knockdown significantly decreases the ErbB4 activity. Importantly, knockdown of ErbB4 can partially rescue the WWP1 siRNA-induced ErbB4 activity increase. These results suggest that WWP1 functions partially through ErbB4 in MCF7.

In addition, HRG-activated ErbB4 has been shown to increase the BRCA1 expression in MCF7 (Muraoka-Cook *et al.*, 2006a). We examined the HRG- β -1-induced BRCA1 mRNA expression by reverse transcriptase-PCR. As shown in Figure 4g, ErbB4 siRNA decreases the BRCA1 mRNA levels by $\sim 20\%$ but WWP1 siRNA increases the BRCA1 mRNA levels by $\sim 30\%$. When we combined both siRNAs, the BRCA1 mRNA level is similar to the control. Consistently, overexpression of WWP1, but not WWP1C890A, decreases the BRCA1 mRNA levels by $\sim 30\%$. These results suggest that the WWP1 knockdown-induced BRCA1 increase is indeed through ErbB4.

Itch and HECW1 also inhibit ErbB4 in breast cancer cell lines

It has been reported that the WWP1 family member, Itch, also targets ErbB4 for degradation in T47D (Omerovic *et al.*, 2007). To test whether the other WWP1 family members also regulate endogenous ErbB4 in breast cancer cells, we knocked down the other eight WWP1 family members by three different siRNAs for each gene in both T47D and MCF7. We found that siRNAs against Itch and HECW1 significantly increase the ErbB4 protein expression in T47D, but not in MCF7 (Figure 5a), although all siRNAs against Itch and HECW1 work well in both cell lines (Figure 5b). We also examined the knockdown efficiencies for several other E3 ligases and found that all siRNAs work well as expected (data not shown). We further compared the mRNA expression of eight E3 ligases in MCF7 and T47D by reverse transcriptase-PCR. As shown in Figure 5c, the Itch and HECW1 mRNA levels are higher in T47D than those in MCF7. These results may explain why knockdown of Itch and HECW1 increase the ErbB4 protein levels in T47D but not in MCF7. Finally, we found that knockdown of WWP1, Itch and HECW1 additively increase the endogenous ErbB4 protein levels in T47D although

WWP1 and Itch are similarly more potent than HECW1 (Figure 5d). These results suggest that Itch and HECW1 also negatively regulate the ErbB4 expression in breast cancer.

The expression of WWP1 and ErbB4 in breast cancer cell lines

As WWP1 negatively regulates the ErbB4 protein stability in breast cancer cell lines, we examined the WWP1 and ErbB4 protein levels in a panel of eight breast cancer cell lines and expected to detect a negative correlation. As shown in Figure 5e, the full-length ErbB4 can only be detected in four ER- α -positive breast cancer cell lines (BT474, MCF7, T47D and HCC1500). Interestingly, WWP1 is also highly expressed in three out of four ER-positive breast cancer cell lines (BT474, MCF7 and HCC1500). These results are consistent with our previous reports (Chen *et al.*, 2007b, 2009). Both ErbB4 and WWP1 are lowly expressed in ER- α -negative breast cancer cell lines (MDA-MB-231, MDA-MB-468, HCC1937 and Hs578T). Thus, there is no negative correlation between the WWP1 protein expression and the ErbB4 protein expression in these breast cancer cell lines.

Discussion

In this study, we provide several lines of evidence to support that WWP1 targets the full-length ErbB4-CYT1 isoforms for ubiquitin-mediated degradation. First, WWP1 binds to ErbB4-CYT1 proteins through the WW/PY motif interaction. Second, WWP1 ubiquitinates ErbB4-CYT1 proteins through its E3 ubiquitin ligase activity. Third, WWP1 decreases the protein half-lives of ErbB4-CYT1 proteins. Most importantly, endogenous WWP1 suppresses the endogenous ErbB4 protein levels and activities in breast cancer.

ErbB4 has been reported to be targeted for degradation by several E3 ligases, including Itch (Omerovic *et al.*, 2007), anaphase-promoting complex/cyclosome (Strunk *et al.*, 2007), and most recently WWP1 (Feng *et al.*, 2009). While our study was ongoing, Feng *et al.* (2009) published that WWP1 selectively targets HER4/ErbB4-JMa-CYT1 and its cleaved m80 for degradation. Although our research results completely support the conclusion that WWP1 targets the full-length ErbB4/HER4 for degradation by using different strategies, there are several new discoveries in our study. First, we identified that the first and third WW domains of WWP1 play major roles for protein interaction. Second, we showed that WWP1 regulates both JMa and JMb isoforms. Most importantly, we examined all WWP1 family members by the RNA interference approach in two breast cancer cell lines and discovered that HECW1 may also suppress ErbB4 in breast cancer. In addition, we found that there is no negative correlation between the expression of WWP1 and ErbB4 in eight breast cancer cell lines. Finally, we found that ErbB4 does not regulate the endogenous WWP1 expression in breast

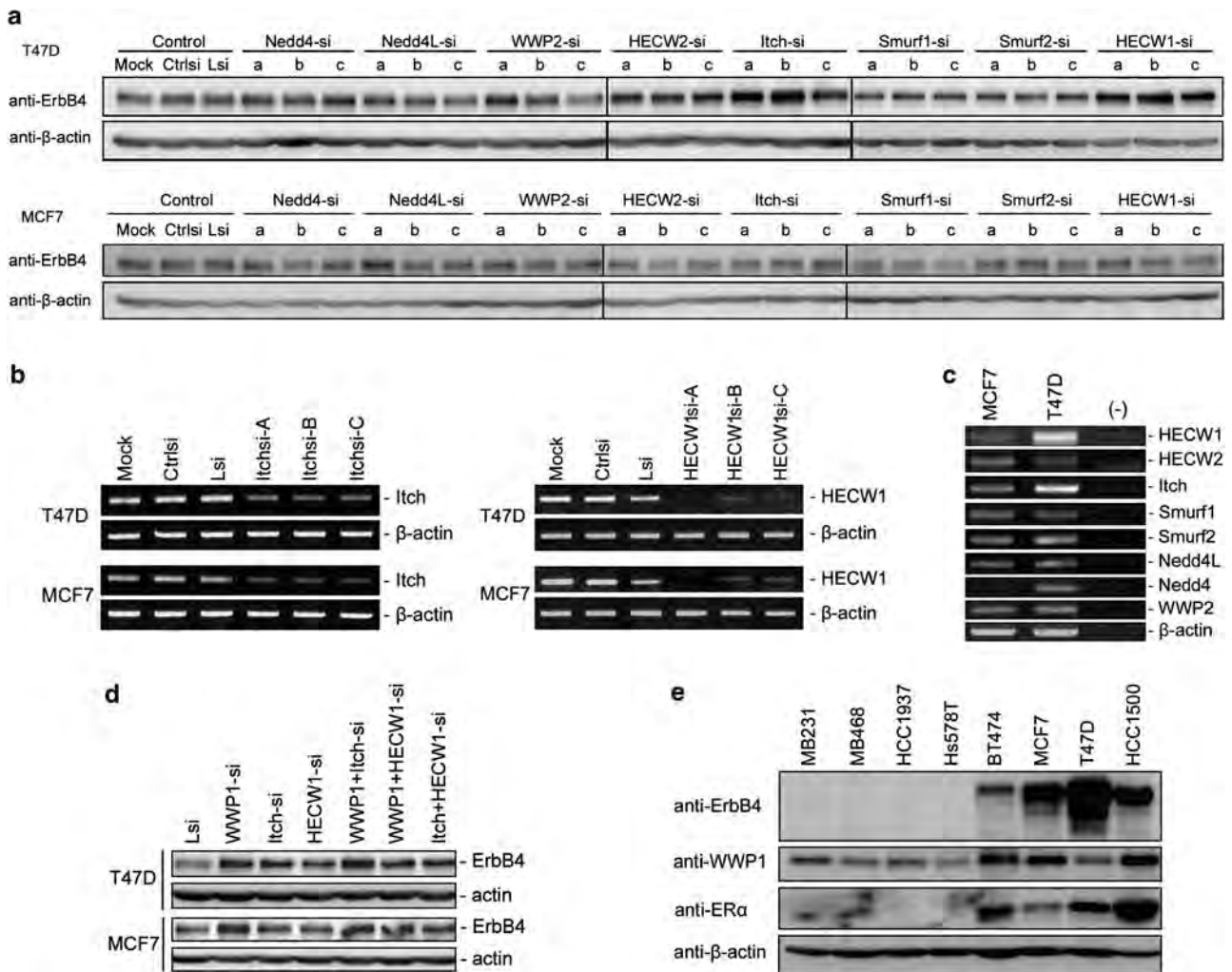


Figure 5 Itch and HECW1 also suppress ErbB4 protein expression and the protein expression of WWP1 and ErbB4 in breast cancer cell lines. **(a)** The screening of the other eight WWP1 family members that suppress the ErbB4 protein expression by RNA interference in T47D and MCF7. The anti-Itch and anti-HECW1 small interfering RNAs (siRNAs) upregulate the ErbB4 protein expression in T47D but not in MCF7. Three different silencer select pre-designed siRNAs for each gene (Ambion) were used to knockdown gene expression at 10 nm final concentration. **(b)** Knockdown efficiencies of anti-Itch and anti-HECW1 siRNAs in T47D and MCF7, as determined by reverse transcriptase (RT)-PCR. **(c)** The mRNA levels of WWP1 family members in MCF7 and T47D, as determined by RT-PCR. **(d)** WWP1, Itch and HECW1 additively inhibit ErbB4 in T47D. **(e)** The expression of ErbB4 and WWP1 was analysed in a panel of breast cancer cell lines by western blotting. Both WWP1 and ErbB4 are predominately expressed in estrogen receptor- α -positive breast cancer cell lines.

cancer (Figure 4e). This is different from the published results that ErbB4 decreases the WWP1 expression (Feng *et al.*, 2009).

We found that WWP1 specifically targets ErbB4-CYT1 but not CYT2 isoforms. Maatta *et al.* (2006) reported that JMa-CYT1, but not JMA-CYT2, is localized to early endosomes. WWP1 is also localized to endosomes (Chen *et al.*, 2008). However, the proteasome inhibitor, MG132, but not the lysosome inhibitor, chloroquine, partially block the WWP1-mediated ErbB4-CYT1 degradation (Figure 3). Interestingly, the combination of MG132 and chloroquine synergistically blocks the WWP1-mediated ErbB4-CYT1 degradation (data not shown). Similar results have been observed by Feng *et al.* (2009). The

degradation mechanism of the ubiquitinated ErbB4 proteins remains to be elucidated.

Although transient WWP1 overexpression does not change the exogenous EGFR, ErbB2 and ErbB3 protein levels in COS7 cells (Feng *et al.*, 2009), we previously found that WWP1 upregulates EGFR and ErbB2 in MCF10A and PC-3 (Chen *et al.*, 2008). As EGFR and ErbB2 do not have a PY motif, the regulation of EGFR and ErbB2 by WWP1 is indirectly mediated through RNF11 (Chen *et al.*, 2008) and may be cell line specific. In contrast, the direct regulation of ErbB4 by WWP1 is broadly detected in multiple cell lines.

One of the primary functions of ErbB4 *in vivo* is in the maturation of mammary glands during pregnancy and

lactation induction (Long *et al.*, 2003; Tidcombe *et al.*, 2003). ErbB4 can activate the expression of the tumor suppressor gene, BRCA1, (Muraoka-Cook *et al.*, 2006a) and differentiation genes such as β -casein (Muraoka-Cook *et al.*, 2008). Most studies suggest that ErbB4 also decreases breast epithelial cell proliferation (Pitfield *et al.*, 2006; Muraoka-Cook *et al.*, 2006a; Feng *et al.*, 2007) and survival (Naresh *et al.*, 2006; Vidal *et al.*, 2007). As an ErbB4-negative regulator, WWP1 has been shown to promote breast epithelial cell proliferation and survival (Chen *et al.*, 2007b, 2009; Nguyen Huu *et al.*, 2008). Whether WWP1 regulates mammary gland development during pregnancy and lactation through suppressing ErbB4 *in vivo* remains to be elucidated by studying the breast-specific WWP1 knockout and WWP1 transgenic mice in the future.

It is surprising that the expression of ErbB4 and WWP1 is not negatively correlated in breast cancer cell lines. Three ER-positive cell lines with ErbB4 expression (BT474, MCF7 and HCC1500) also show higher levels of WWP1 compared with four ER-negative breast cancer cell lines (Figure 5e). The WWP1 protein expression has been associated with positive ER- α and good-prognosis breast tumors (Nguyen Huu *et al.*, 2008; Chen *et al.*, 2009). Consistently, most publications associated the ErbB4 protein expression with ER- α -positive, low grade and more differentiated breast tumors (Tang *et al.*, 1999; Suo *et al.*, 2001; Witton *et al.*, 2003; Junttila *et al.*, 2005). However, knockdown of endogenous WWP1 significantly upregulates endogenous ErbB4 protein expression in both T47D and MCF7 (Figure 4a). It is becoming evident that the expression of ErbB4 is dynamically controlled by synthesis and degradation, which may explain the observation that both WWP1 and ErbB4 are co-expressed in the same breast cancer cells.

Besides WWP1, another WWP1 family member, Itch, has been reported to target ErbB4 for ubiquitin-mediated degradation (Omerovic *et al.*, 2007). We confirmed that knockdown of Itch can upregulate ErbB4 in T47D but not in MCF7 (Figure 5). It is partially because Itch is lowly expressed in MCF7. Similar results were observed for HECW1. HECW1 (NEDL1) has been shown to be an E3 ligase for Dvl-1 (Miyazaki *et al.*, 2004) and to cooperate with p53 to induce apoptosis independent of its E3 ligase activity (Li *et al.*, 2008a). Thus, WWP1, Itch and HECW1 additively suppress the ErbB4 expression in some breast cancers.

In summary, we show that WWP1 specifically targets the ErbB4-CYT1 proteins for ubiquitin-mediated degradation and regulates its biological activities in breast cancer. Given the frequent gene amplification and overexpression of WWP1 in prostate and breast cancer, this knowledge may help us understand the role of WWP1 in prostate and breast cancer development and may be useful for guiding the development of anti-WWP1 and pan-ErbB kinase inhibitors for future prostate and breast cancer target therapy.

Materials and methods

Cell culture and transfection

The human embryonic kidney 293T (HEK293T) cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PS). MCF7 breast cancer cells were grown in DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acid, 1.5 g/l sodium bicarbonate, 0.01 mg/ml insulin, 5% FBS and 1% PS. T47D breast cancer cells were cultured in RPMI 1640 medium with 2 mM L-glutamine and supplemented with 1 mM sodium pyruvate, 4.5 g/l glucose, 10 mM HEPES, 1.5 g/l sodium bicarbonate, 5% FBS, 0.01 mg/ml insulin and 1% PS.

All transient transfections for plasmids and siRNAs were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Two chemically synthesized WWP1 siRNAs were purchased from Dharmacon (Chicago, IL, USA) and transfected at 100 nM final concentration. The siRNA target sequences for human WWP1 gene are 5'-GAAGTCATCTGTAACTAAA-3' (Wsi#1) and 5'-GCAGAGAAATACTGTTTAT-3' (Wsi#2). The 3'-UU overhang for both strands and 5'-phosphorylation for antisense sequence were used for these siRNAs. The silencer select pre-designed siRNAs for ErbB4 and eight WWP1 family members were purchased from Applied Biosystems (Ambion, Austin, TX, USA). The target sequences for ErbB4 siRNAs are 5'-CCCTTACAATGCAATGGAATT-3' and 5'-CCCCTAATGTCTTAGTGAATT-3'. The final concentration for silencer select siRNAs was 10 nM.

Expression plasmids

The plasmids expressing WT WWP1, the catalytic inactive hWWP1C890A, mWWP1C886S, and Myc-Ub have been described in our previous studies (Chen *et al.*, 2005, 2007b). The ErbB4-JMa-CYT1, JMa-CYT2, JMb-CYT1, JMb-CYT1-PY3 mutant and pSRE-Luc constructs have been described in a previous study (Omerovic *et al.*, 2007). The first and second PY motifs were mutated based on the JMb-CYT1-PY3 mutant using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The resulting constructs express ErbB4-JMb-CYT1-PY1, 3 and ErbB4-JMb-CYT1-PY2, 3.

Antibodies

The anti-WWP1 rabbit polyclonal Ab has been described in our previous report (Chen *et al.*, 2007a). The anti-WWP1 mouse monoclonal Ab (1A7) is from Novus Biologicals, Inc (Littleton, CO, USA). The anti- β -actin mouse monoclonal Ab AC-15 (#A5441) is from Sigma (St Louis, MO, USA). The anti-Myc mouse monoclonal Ab 9B11 (#2276) is from Cell Signaling (Danvers, MA, USA). The anti-ErbB4 rabbit polyclonal Ab (C-18, #Sc-283), anti-ER- α Ab (H-184, #Sc-7207), anti-Ub Ab (#Sc-8017) and the rabbit IgG (#Sc-2027) are from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunoprecipitation and GST pull-down

Immunoprecipitation using an anti-Myc Ab plus protein A-agarose beads and GST pull-down experiments have been described in our previous studies (Chen *et al.*, 2005; Li *et al.*, 2008b). The anti-ErbB4 rabbit polyclonal Ab was used to immunoprecipitate the endogenous ErbB4 proteins from MCF7. The rabbit IgG was used as the negative control. The beads were washed five times with 500 μ l of 1 \times cell lysis buffer. Proteins were resuspended with 30 μ l of SDS sample loading buffer and analysed by western blot.

Protein ubiquitination assay

HEK293T cells were transiently transfected with Myc-Ub and other plasmids as necessary in 6-well plates. Two days after

Table 1 Primer sequences for reverse transcriptase-PCR

Gene	Primer	Sequence (5'→3')
HECW1	Forward	TCCTTCCTGAGGCTGAAC
	Backward	CTGTTGCAATGGTTCGCTGA
Itch	Forward	AGCTGAGTGGAGGTTGTCTC
	Backward	GCAATCGGCA GGTCCAGTA
Smurf1	Forward	TTGGCTTTGGTCACACAGG
	Backward	GCTGGGATGTGAAATCTGGA
Smurf2	Forward	ACACTTGCTTCAATCGAATA
	Backward	AGGAGGCTGTCTAGTCAGGGTT
Nedd4	Forward	TGCCAAGAGCTCATACCTGT
	Backward	GGGAAGACTCAGTGGCCACA
Nedd4L	Forward	TCACAGGGACATCGCGAGT
	Backward	TCTGCCATCGCCTCTGCAA
WWP2	Forward	ACATCATGCTGCTGACTGAC
	Backward	GCTCATGTCTATCTCTGCA
BRCA1	Forward	CATCATTCACCCTTGGCACA
	Backward	TGGCTGCAGTCAGTAGTGGC
β-actin	Forward	GAAATCGTGCCTGACATTAAG
	Backward	CTAGAAGCATTTCGGTGGACGATGGAGGGGCC

transfection, the cells were harvested in 150 µl SDS lysis buffer (50 mM Tris-Cl, pH 6.8, 1.5% SDS). The samples were boiled for 15 min. One hundred µl of protein lysate was diluted with 1.2 ml EBC/bovine serum albumin (BSA) buffer (50 mM Tris-Cl, pH 6.8, 180 mM NaCl, 0.5% CA630, 0.5% BSA) and incubated with 30 µl 50% anti-ErbB4 Ab together with protein A/G Plus-Agarose (#Sc-2003, Santa Cruz Biotechnology) overnight at 4 °C with rotation. The beads were collected by centrifugation at 10 000 g for 30 s at 4 °C and washed three times with 1 ml ice-cold EBC/BSA buffer. Proteins were resuspended with 30 µl of SDS sample loading buffer and analysed by western blot. Ubiquitin-conjugated ErbB4 was detected by anti-Myc Ab or anti-Ub Ab.

Luciferase reporter assay

MCF7 cells were transfected with the SRE-luciferase reporter and the indicated combinations of WWP1 siRNA#1 and ErbB4 siRNA#1. At 8 h after transfection, the cells were starved overnight (16 h) and stimulated with 100 ng/ml of HRG-β1 (Sigma) for 8 h. Luciferase activities were measured by using the luciferase reporter assay system with a GloMax 20/20 Luminometer (Promega, Madison, WI, USA).

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Reverse transcriptase-PCR

Total RNA was extracted from the MCF7 and T47D cells using the Trizol reagent (Invitrogen). The cDNA was prepared by using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The primers used in this study are shown in Table 1.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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KLF5 Promotes Breast Cell Survival Partially through Fibroblast Growth Factor-binding Protein 1-pERK-mediated Dual Specificity MKP-1 Protein Phosphorylation and Stabilization*

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Rong Liu[†], Han-Qiu Zheng[†], Zhongmei Zhou[†], Jin-Tang Dong[§], and Ceshi Chen^{†1}

From the [†]Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York 12208 and the [§]Winship Cancer Institute and Department of Hematology and Oncology, Emory University School of Medicine, Atlanta, Georgia 30322

Krüppel-like transcription factor 5 (*KLF5*) is a zinc-finger transcription factor promoting cell survival and tumorigenesis in multiple cancers. A high expression level of *KLF5* has been shown to be associated with shorter breast cancer patient survival. However, the role of *KLF5* and mechanism of *KLF5* actions in breast cancer remain unclear. In this study, we found that *KLF5* knockdown by small interfering RNA in two breast cell lines, MCF10A and BT20, induces apoptosis. Interestingly, a pro-survival phosphatase, dual specificity mitogen-activated protein kinase phosphatase 1 (MKP-1), is down-regulated by *KLF5* ablation. Consistently, *KLF5* overexpression increases the MKP-1 protein expression in Hs578T and MCF7. We further found that MKP-1 is essential and sufficient for *KLF5* to promote breast cell survival. However, *MKP-1* is not a *KLF5* direct transcription target because the *MKP-1* mRNA level is not regulated by *KLF5*. By cycloheximide chase assays, we found that *KLF5* decreases MKP-1 protein degradation via activating the ERK signaling. Inhibition of pERK by the pharmacological inhibitor U0126 specifically blocks *KLF5*-induced MKP-1 phosphorylation and stabilization. Additionally, constitutive activation of ERK by constitutively activated MEK1 rescues the *KLF5* depletion-induced MKP-1 down-regulation. Consistently, the phosphorylation-deficient MKP-1 mutant cannot be stabilized by *KLF5*. Finally, the activation of ERK by *KLF5* is very likely through the *KLF5* direct target gene *FGF-BP* in breast cells. These findings suggest that *KLF5* is a pro-survival factor that promotes breast cell survival partially through pERK-mediated MKP-1 phosphorylation and stabilization. The *KLF5*-*FGF-BP*-pERK-MKP-1 signaling axis may provide new therapeutic targets for invasive breast cancer.

The Krüppel-like transcription factor 5 (*KLF5/IKLF/BTB2*)² has been suggested to be an oncogene in multiple

carcinomas including the intestinal (1), esophageal (2), bladder (3), and breast (4). A high level of the *KLF5* mRNA has been reported to associate with a short survival time in breast cancer patients (4). In addition, *KLF5* expression is induced by a number of oncogenes including *ERBB2* (5), *RAS* (6), and *WNT* (7). Consistently, *KLF5* has been shown to promote cell proliferation (3), migration (8), and tumorigenesis (3) in different cell models by regulating gene transcription. *KLF5* has been shown to promote cell survival through regulating Survivin (9), Pim1 (10), and PARP1 (11) in different types of cells.

Our previous study showed that *KLF5* promotes the TSU-Pr1 bladder cancer cell growth *in vitro* and *in vivo* (3). Furthermore, we demonstrated that *KLF5* regulates a number of downstream target genes in a microarray study. Following that, we proved that *KLF5* promotes breast cell proliferation partially through directly inducing the fibroblast growth factor-binding protein 1 (*FGF-BP*) transcription in breast cancer.³ *FGF-BP* was confirmed to be a *KLF5*-induced gene in the mouse lung in an independent microarray study (13).

Besides *FGF-BP*, another *KLF5* downstream target gene (3), dual specificity mitogen-activated protein kinase phosphatase 1 (*MKP-1/DUSP1/CL-100*), has been documented to promote cell survival (14). Mitogen-activated protein kinases (MAPKs) are activated via phosphorylation of ERK, p38, and JNK. These MAPKs are inactivated via de-phosphorylation by MKPs including MKP-1 (15). Although pERK usually contributes to cell proliferation and survival, pJNK and pp38 promote cell apoptosis in response to stress (16). The balance between MAPKs and MKPs determines whether cells undergo survival or apoptosis (17). Consistently, MKP-1 has been reported to be overexpressed in many types of cancer including breast cancer (15, 18). It has been shown that MKP-1 is rapidly induced in response to multiple stress stimuli, such as the chemotherapy drugs paclitaxel (14) and cisplatin (19, 20), oxidative stress (21), and UV radiation (22), and contributes to cell survival. The MKP-1 induction by stress is at both transcriptional (23, 24) and post-translational (25, 26) levels and primarily mediated by the activation of ERK signaling. Interestingly, the pERK levels are increased by *KLF5* in TSU-Pr1 (3).

Here, we studied the mechanism by which MKP-1 is induced by *KLF5* in breast cancer. We showed evidence that *KLF5* pro-

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¹ To whom correspondence should be addressed. E-mail: chenc@mail.amc.edu.

² The abbreviations used are: *KLF5*, Krüppel-like factor 5; ERK, extracellular signal-regulated kinase; WT, wild type; MKP-1, dual specificity mitogen-activated protein kinase phosphatase 1; *FGF-BP*, fibroblast growth factor-binding protein 1; PARP1, poly(ADP-ribose) polymerase 1; siRNA, small interfering RNA; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH₂-terminal kinase; CHX, cycloheximide.

³ H. Q. Zheng, Z. Zhou, L. Chaudhury, J. T. Dong, and C. Chen, unpublished data.

motes breast cell survival partially through MKP-1. The induction of MKP-1 by KLF5 in breast cells is at the protein post-translational level but not the transcriptional level. The activation of ERK signaling by KLF5 is essential and sufficient for MKP-1 protein phosphorylation and stabilization in breast cells. We further demonstrated that activation of ERK signaling is likely mediated by the KLF5 direct target gene *FGF-BP*. Taken together, the KLF5-FGF-BP-pERK-MKP-1 signaling axis may contribute to breast cancer and provide new therapeutic targets for breast cancer.

MATERIALS AND METHODS

Breast Cell Lines and Culture Conditions—The immortalized breast epithelial cell line MCF10A was maintained in Dulbecco's modified Eagle's medium/Ham's F-12 50/50 medium supplemented with 5% horse serum, 0.5 μ g/ml hydrocortisone, 10 μ g/ml insulin, 20 ng/ml epidermal growth factor, 0.1 μ g/ml cholera enterotoxin, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. The breast cancer cell lines BT-20 and MCF7 were cultured in minimal essential medium containing 5% fetal bovine serum, 0.1 mM non-essential amino acid, 1.5 g/liter sodium bicarbonate, 1 mM sodium pyruvate, 0.01 mg/ml insulin, and 100 units/ml penicillin and 100 μ g/ml streptomycin. The breast cancer cell line Hs578T was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1.5 g/liter sodium bicarbonate, 1 mM sodium pyruvate, 0.01 mg/ml insulin, and 100 units/ml penicillin and 100 μ g/ml streptomycin. These cells were maintained in a humidified atmosphere with 5% CO₂ at 37 °C.

Immunoblotting and Antibodies—Immunoblotting was performed with 40 μ g of proteins. The anti- β -actin and anti-V5 antibodies are from Sigma. The anti-PARP, anti-cleaved caspase 3, anti-pERK, and anti-pMKP-1^{Ser-359} antibodies are from Cell Signaling (Danvers, MA). The anti-KLF5 rabbit polyclonal antibody has been described previously (27). The anti-MKP-1 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

siRNA Transfection and Adenovirus Infection—The control *luciferase* siRNA (Lucsi), *KLF5* siRNA (KLF5si) (Dharmacon, Chicago, IL), and *MKP-1* siRNA (MKP-1si) (silencer select pre-designed siRNA, Ambion, Austin, TX) were transfected by Lipofectamine 2000 (Invitrogen). The siRNA target sequences were: 5'-AGCTCACCTGAGGACTCACAC-3', for the human *KLF5* gene, 5'-CTTACGCTGAGTACTTCGA-3' for the *luciferase* gene, and 5'-GGACTAATCGAGTCAAGCT-3' for the human *MKP-1* gene. The final concentration of Lucsi and KLF5si was 100 nM; and the final concentration of MKP-1si was 10 nM.

The *KLF5* and control *gfp* adenoviruses have been described previously (3). MCF7 and Hs578T cells were infected with adenoviruses in media containing 5% fetal bovine serum. After incubation with the adenoviruses for 4 h, the cells were cultured in normal growth media.

Cycloheximide (CHX) Chase Assays—Hs578T, MCF10A, and HEK293T cells were seeded into a 12-well plate at a density of $1\text{--}2.5 \times 10^5$ cells per well. After overnight culture, the cells were either transfected with different siRNAs or plasmids or

infected with adenoviruses. Two days after transfection or infection, the cells were treated with 50 μ g/ml CHX. Total proteins were collected at different time points and subjected to immunoblotting for KLF5, MKP-1, and β -actin.

Reverse Transcriptase-PCR—Total RNAs were isolated using TRIzol[®] reagent (Invitrogen). Reverse transcriptions were performed using the Iscript[™] cDNA synthesis kit (Bio-Rad). Forward primer, 5'-GATCTAGATATGCCAGTTC-3', and reverse primer, 5'-CAGCCTTCCCAGGTACACTTG-3', were used to amplify *KLF5* by PCR in a 20- μ l volume. Primer sequences for *MKP-1* were 5'-CCCGGAGCTGTGCAGCAA-3' (forward) and 5'-CTGGCCCATGAAGCTGAAGT-3' (reverse). A total of 32 cycles were used to amplify *KLF5* and *MKP-1*, whereas 28 cycles were used to amplify the β -actin control.

Cell Viability Assay—MCF10A and BT20 cells were transfected with KLF5si, MKP-1si, and Lucsi, respectively, for 5 days before analysis. The SRB assay was used to measure cell viability as described in our previous report (28).

Plasmids and Gene Overexpression by Lentiviruses—The human *MKP-1* gene was amplified from IMAGE clone 5296005 with the pfu enzymes by PCR using primers 5'-ttggatccATGGT-CATGGAAGTGGGCAC-3' and 5'-ttctcgagTCAGCAGCTGGGAGAGGTCG-3'. The catalytically inactive *MKP-1*^{S359A/S364A} mutant was generated by PCR using primers 5'-GTTTGTCCACTCCCAGGCAGGCATTTCCCG-3' and 5'-TGCCTGCCTGGGAGTGGACAAACACCCTTC-3'. The *MKP-1*^{S359A/S364A} mutant was generated by primers 5'-ttggatccATGGTTCATGGAAGTGGGCAC-3' and 5'-ttctcgagTCAGCAGCTGGGTGCGGTCGTAATGGGTGCTGAAGGTAGCTCAGCGCAC-3'. The PCR products were digested by BamHI/XhoI and subcloned into the pLenti6/V5-D-TOPO vector and verified by DNA sequencing. The pLenti6/V5-GW/*lacZ* vector (Invitrogen) was used as a negative control.

A constitutively activated MEK1 was amplified from *pMCL-MEK1- Δ ED* (29) (a gift from Dr. A. E. Aplin, Thomas Jefferson University, Philadelphia, PA) and subcloned into pLenti6/V5-D-TOPO vector. All plasmids were transfected into HEK 293FT packing cells using Lipofectamine 2000. Lentiviruses were collected at 72 h after transfection and used to transduce MCF10A cells in a 6-well plate. Forty-eight h after transduction, the antibiotic blasticidin (10 μ g/ml) was added to select drug-resistant populations.

RESULTS

KLF5 Knockdown Induces Apoptosis and Decreases the MKP-1 Expression in Breast Cells—KLF5 has previously been shown to express in estrogen receptor α negative basal-like breast cells.³ To determine whether KLF5 promotes breast cell survival, we knocked down KLF5 in two KLF5 positive breast cell lines, MCF10A and BT20 (30). We examined the levels of apoptosis markers, cleaved PARP, and caspase 3, in the control *luciferase* siRNA (Lucsi) and well characterized *KLF5* siRNA (KLF5si) (3, 31) transfected cells by immunoblotting. We found that KLF5si induces the cleavage of both PARP and caspase 3 compared with Lucsi in MCF10A and BT20 (Fig. 1A). To further confirm that KLF5 knockdown decreases cell survival through inducing apoptosis, we measured cell viability by the

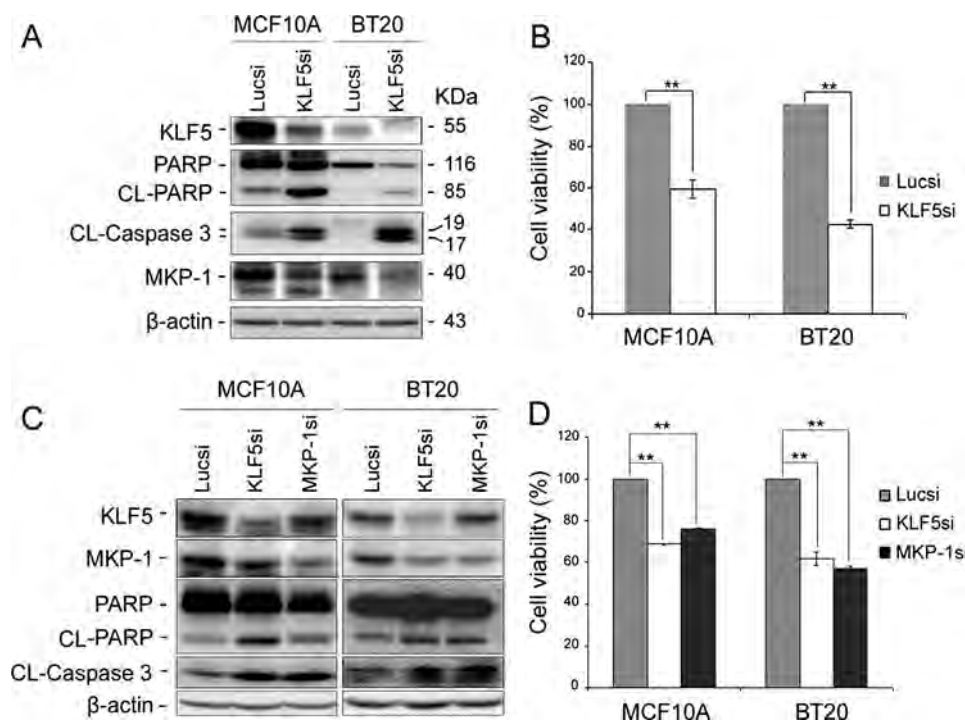


FIGURE 1. Knockdown of KLF5 induces apoptosis and down-regulates the MKP-1 protein levels in breast cells. A, knockdown of KLF5 induces the PARP and caspase 3 cleavage and down-regulates the MKP-1 protein levels in MCF10A and BT20. A well characterized *KLF5* siRNA was used to knockdown the KLF5 expression in MCF10A and BT20 cells. Luciferase siRNA (Lucsi) was used as the negative control. Protein levels were detected by immunoblotting. B, *KLF5* siRNA significantly reduces cell viability in MCF10A and BT20 as determined by the SRB assay. **, $p \leq 0.001$ (*t* test). C, knockdown of MKP-1 induces the PARP and caspase 3 cleavage in both MCF10A and BT20 cell lines compared with the Lucsi negative control and the KLF5si positive control. D, the *MKP-1* siRNA significantly reduces cell viability in MCF10A and BT20 compared with the Lucsi negative control and the KLF5si positive control. Data are presented as the mean \pm S.D. (error bars) from three independent experiments.

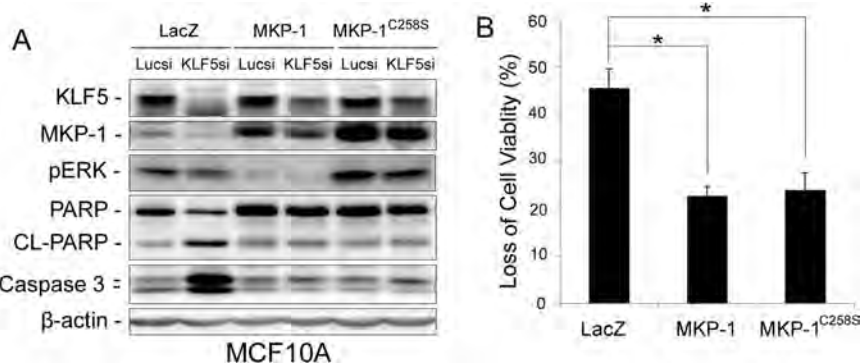


FIGURE 2. MKP-1 partially rescues the KLF5 knockdown-induced apoptosis in MCF10A. A, overexpression of either WT MKP-1 or the catalytically inactive MKP-1^{C258S} mutant decreases the *KLF5* siRNA-induced PARP and caspase 3 cleavage. MCF10A cell populations stably expressing LacZ, MKP-1, or MKP-1^{C258S} were transfected with Lucsi or KLF5si for 4 days. The apoptosis markers including cleaved PARP and caspase 3 were measured by immunoblotting. B, overexpression of MKP-1 or MKP-1^{C258S} significantly decreases the *KLF5* knockdown induced loss of cell viability as shown by the SRB assay. *, $p < 0.05$ (*t* test). Data are presented as the mean \pm S.D. (error bars) from three independent experiments.

SRB assay and Annexin V levels by flow cytometry. Consistent with Western blot results, KLF5si significantly decreases cell viability (Fig. 1B) and increases Annexin V staining (data not shown) in both MCF10A and BT20. Interestingly, the protein expression levels of a potential KLF5 downstream gene, the pro-survival phosphatase MKP-1, are decreased by KLF5si in both cell lines (Fig. 1A).

To test if MKP-1 indeed promotes breast cell survival, we knocked down MKP-1 by a pre-designed anti-MKP-1 siRNA in

both MCF10A and BT20 and examined apoptosis. As expected, knockdown of MKP-1 also induces the cleavage of both PARP and caspase 3 and the decrease of cell viability like knockdown of KLF5 in both MCF10A and BT20 (Fig. 1, C and D).

KLF5 Promotes Cell Survival Partially through MKP-1—Because silence of KLF5 induces apoptosis and down-regulates the expression of the pro-survival MKP-1 protein in breast cells, we wondered if KLF5 functions partially through MKP-1. We performed a rescue experiment in MCF10A to determine whether MKP-1 overexpression can block the KLF5si-induced apoptosis. The wild-type (WT) MKP-1, the catalytically inactive mutant *MKP-1*^{C258S} (32), and the *lacZ* control genes were forced overexpressed in MCF10A populations, respectively, by lentiviruses (Fig. 2A). In line with our previous observation, KLF5si decreases the MKP-1 protein level and induces apoptosis, indicated by cleavage of PARP and caspase 3 and loss of cell viability, in the control LacZ overexpressing cells. As expected, forced overexpression of WT MKP-1 clearly decreases the pERK levels and KLF5si-induced apoptosis (Fig. 2). Similar results were obtained from two stable MKP-1 overexpressing MCF10A clones (data not shown). Unexpectedly, overexpression of the catalytically inactive mutant *MKP-1*^{C258S} also blocks the KLF5si-induced apoptosis as efficiently as WT MKP-1. As a dominant negative MKP-1 mutant, MKP-1^{C258S} increases the pERK levels (Fig. 2A). Consistently, the expression level of MKP-1^{C258S} is higher than that of WT MKP-1 presumably because a high level of pERK stabilizes the MKP-1 protein (see below in detail). These findings

suggest that overexpression of MKP-1 can partially rescue the KLF5si-induced apoptosis in MCF10A.

MKP-1 Expression Is Positively Regulated by KLF5 at the Protein Level but Not at the mRNA Level in Breast Cells—KLF5 is a well established transcriptional factor regulating transcription of a number of genes. To test whether *MKP-1* is a KLF5 direct transcriptional target, we examined *MKP-1* expression at the protein level by Western blot and the mRNA level by semi-quantitative reverse transcriptase-PCR after knocking down

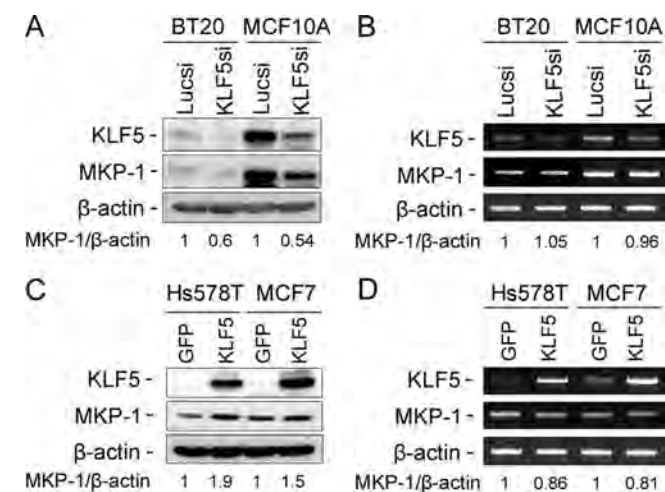


FIGURE 3. KLF5 up-regulates the MKP-1 expression at the protein level but not at the mRNA level. KLF5 siRNA decreases the MKP-1 protein levels (A) but not mRNA levels (B) in MCF10A and BT20 as measured by immunoblotting and semi-quantitative reverse transcriptase-PCR, respectively. Lucsi was used as a negative control. β -Actin served as the input control. KLF5 overexpression by adenoviruses increases the MKP-1 protein levels (C) but not mRNA levels (D) in Hs578T and MCF7 breast cancer cells. The *gfp* adenovirus was used as a negative control. The normalized band intensities are shown below each lane (the negative controls are defined as 1). GFP, green fluorescent protein.

and overexpressing KLF5. To our surprise, KLF5si decreases *MKP-1* expression at the protein level but not at the mRNA level in both MCF10A and BT20 cell lines (Fig. 3, A and B). Additionally, KLF5 overexpression increases the expression of *MKP-1* at the protein level but not at the mRNA level in both Hs578T and MCF7 (Fig. 3, C and D). Finally, we found that KLF5 cannot activate the *MKP-1* promoter in MCF7 by dual luciferase reporter assays (data not shown). These results suggest that *MKP-1* is not a KLF5 direct transcription target gene in breast cells.

MKP-1 is a short-lived protein (the half-life is about 45 min in fibroblasts) degraded through the ubiquitin proteasome pathway (25). To investigate whether KLF5 regulates MKP-1 protein stability, we performed CHX chase assays and found that KLF5 knockdown in MCF10A cells decreases the endogenous MKP-1 protein half-life (Fig. 4, A and B). Consistently, KLF5 overexpression clearly increases the MKP-1 protein half-life in Hs578T (Fig. 4, C and D). These results suggest that KLF5 decreases MKP-1 protein degradation in breast cells.

KLF5 Increases MKP-1 Protein Stability through the pERK-mediated MKP-1 Phosphorylation—The proteasomal degradation of MKP-1 has been demonstrated to be inhibited by pERK-mediated MKP-1 phosphorylation at Ser-359 and Ser-364

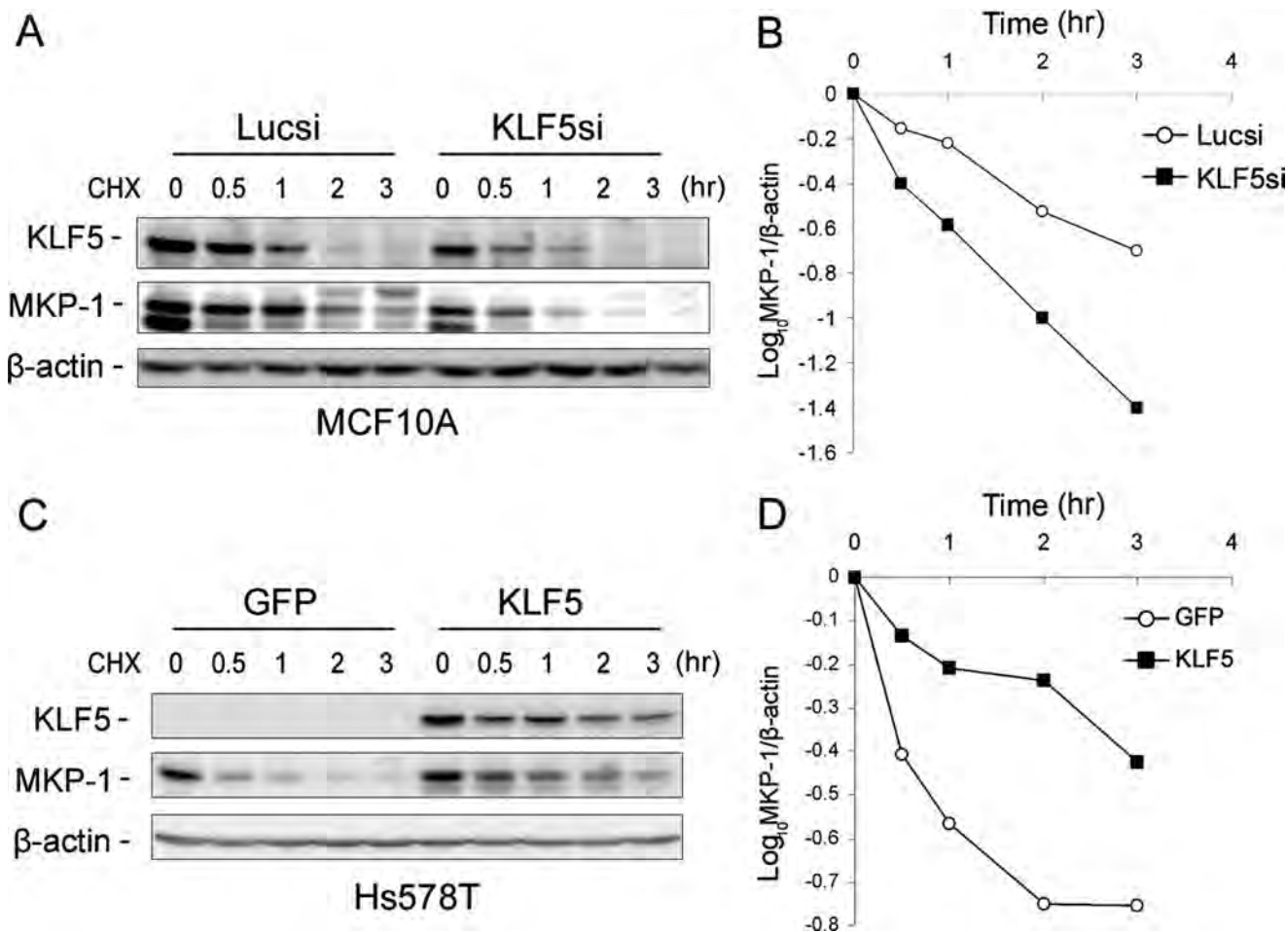


FIGURE 4. KLF5 increases the MKP-1 protein stability in breast cells. A and B, knockdown of KLF5 decreases the protein half-life for MKP-1 in MCF10A as determined by the CHX (50 μ g/ml) chase assay. The band intensities were quantified using densitometry. C and D, overexpression of KLF5 increases the half-life of the MKP-1 protein in Hs578T as determined by the CHX chase assay. The cells were infected with Ad-KLF5/*gfp* and Ad-*gfp* control adenoviruses, respectively. GFP, green fluorescent protein.

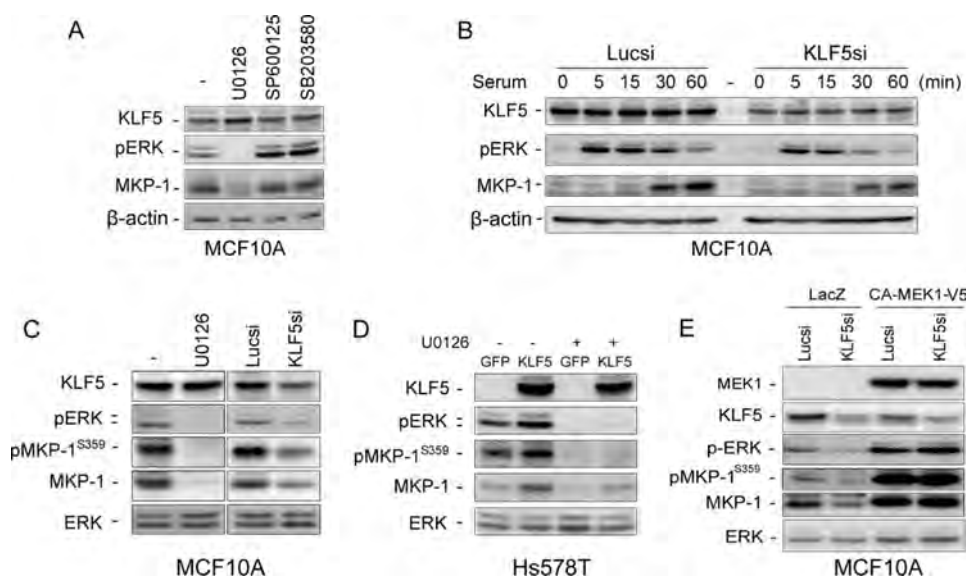


FIGURE 5. KLF5 increases MKP-1 protein stability through activating the ERK signaling in breast cells. A, inhibition of ERK but not JNK and p38 by pharmacological inhibitors reduces the MKP-1 protein level in MCF10A. The cells were serum starved overnight before serum stimulation for 1 h. Dimethyl sulfoxide, U0126 (5 nM, a MEK inhibitor), SP600125 (20 nM, a JNK inhibitor), and SB203580 (20 nM, a p38 inhibitor) were added separately 1 h before serum stimulation. B, knockdown of KLF5 decreases pERK activation and MKP-1 induction by serum. MCF10A cells transfected with KLF5 siRNA and luciferase siRNA were serum starved overnight and cultured in serum-containing media for the indicated time. C, either inhibition of pERK by U0126 or knockdown of KLF5 clearly reduce the pMKP-1^{Ser-359} and total MKP-1 levels in MCF10A. D, the activation of ERK is essential for KLF5 to up-regulate the pMKP-1^{Ser-359} and total MKP-1 levels in Hs578T. Hs578T cells were infected with Ad-KLF5/gfp and Ad-gfp control adenoviruses, respectively. The cells were treated with either dimethyl sulfoxide or U0126 (5 nM) as indicated. E, constitutively activated MEK1 (V5 tagged) rescues KLF5 knockdown-induced down-regulation of pMKP-1^{Ser-359} and total MKP-1. The total ERK level serves as the loading control. GFP, green fluorescent protein.

residues (25). Additionally, the SCF^{SKP2} E3 ubiquitin ligase has been suggested to promote MKP-1 ubiquitin-mediated degradation (26). We first found that KLF5si does not increase the SKP2 expression levels in MCF10A and BT20 (data not shown). Then, we determined whether KLF5 stabilizes MKP-1 via pERK-mediated MKP-1 phosphorylation because KLF5 up-regulates the pERK levels in TSU-Pr1 (3) and KLF5si decreases the pERK levels in MCF10A (Fig. 2A). Indeed, inhibition of pERK, but not JNK and p38, by pharmacological inhibitors dramatically decreases the MKP-1 protein level in MCF10A (Fig. 5A). Consistently, KLF5si reduces the serum-induced pERK and MKP-1 levels in MCF10A (Fig. 5B). Furthermore, we demonstrated that both the MEK inhibitor U0126 and KLF5si dramatically decrease pERK levels and MKP-1 phosphorylation at Ser-359 by using an antibody that specifically recognizes the pMKP-1^{Ser-359} (Fig. 5C). Additionally, we found that KLF5 overexpression up-regulates pERK, pMKP-1^{Ser-359}, and total MKP-1 levels in Hs578T. When pERK is inhibited by U0126, KLF5 overexpression fails to increase the levels of pMKP-1^{Ser-359} and total MKP-1 in Hs578T (Fig. 5D). Finally, the constitutive activation of ERK by overexpression of constitutively activated MEK1 can completely rescue the KLF5si-induced pMKP-1^{Ser-359} and total MKP-1 down-regulation in MCF10A (Fig. 5E). These results strongly suggest that KLF5 stabilizes MKP-1 through activation of ERK.

To further confirm that phosphorylation of MKP-1 at Ser-359/Ser-364 is essential for KLF5-mediated MKP-1 protein stabilization, we generated a MKP-1 mutant in which both Ser-359 and Ser-364 residues were replaced with Ala. As shown in

Fig. 6, A and B, WT MKP-1 can be stabilized by KLF5 overexpression in HEK293T cells. However, the MKP-1^{S359A/S364A} mutant cannot be stabilized by KLF5 under the same conditions. Consistently, the mutant MKP-1 shows a shorter half-life than WT MKP-1 presumably due to lack of phosphorylation at Ser-359 and Ser-364 by pERK (Fig. 6, C and D). The degradation of MKP-1^{S359A/S364A} is still through proteasome because the proteasome inhibitor MG132 can dramatically protect it from degradation in HEK293T cells (Fig. 6E).

A recent study in our laboratory showed that KLF5 directly regulates transcription of the *FGF-BP* in breast cells.³ Because FGF-BP has been reported to activate ERK signaling (33), we hypothesized that KLF5 up-regulates the pERK level through FGF-BP. To test this hypothesis, we knocked down both KLF5 and FGF-BP individually in MCF10A. As shown in Fig. 7A, knockdown of either KLF5 or FGF-BP decreases the pERK,

pMKP-1^{Ser-359}, and total MKP-1 levels. Additionally, we found that KLF5 cannot increase the MKP-1 protein level in the presence of FGF-BP siRNA in MCF7 (Fig. 7B). Thus, KLF5 may activate ERK-MKP-1 signaling through FGF-BP in breast cells (Fig. 7C).

DISCUSSION

Accumulated evidence suggests that KLF5 is a pro-survival factor. First, KLF5 has been shown to promote leukemia cell survival through directly inducing the Survivin gene expression (9). Second, KLF5 was reported to promote cell survival by directly promoting the survival kinase Pim1 expression in the HCT116 colon cancer cell line (10). In addition, KLF5 promotes HeLa and NIH-3T3 cell survival from tumor necrosis factor- α through interacting with PARP1 (11). Finally, the cardiovascular apoptosis is significantly increased in KLF5 heterozygous knock-out mice (11). High expression levels of KLF5 mRNA have been shown to be a prognostic factor for shorter disease-free survival and overall survival in patients with breast cancer (4). Our previous studies suggest that KLF5 is expressed in immortalized breast epithelial cell lines and a subset of estrogen receptor negative breast cancer cell lines (27, 30).³ However, the role of KLF5 and the mechanism by which KLF5 functions in the breast have not been well studied.

In this study, we showed that KLF5 promotes breast cell survival through the FGF-BP-pERK-MKP-1 signaling axis. First, we demonstrated that depletion of endogenous KLF5 or MKP-1 in two breast cell lines, MCF10A and BT20, induces apoptosis (Fig. 1). Next, we found that KLF5 maintains and

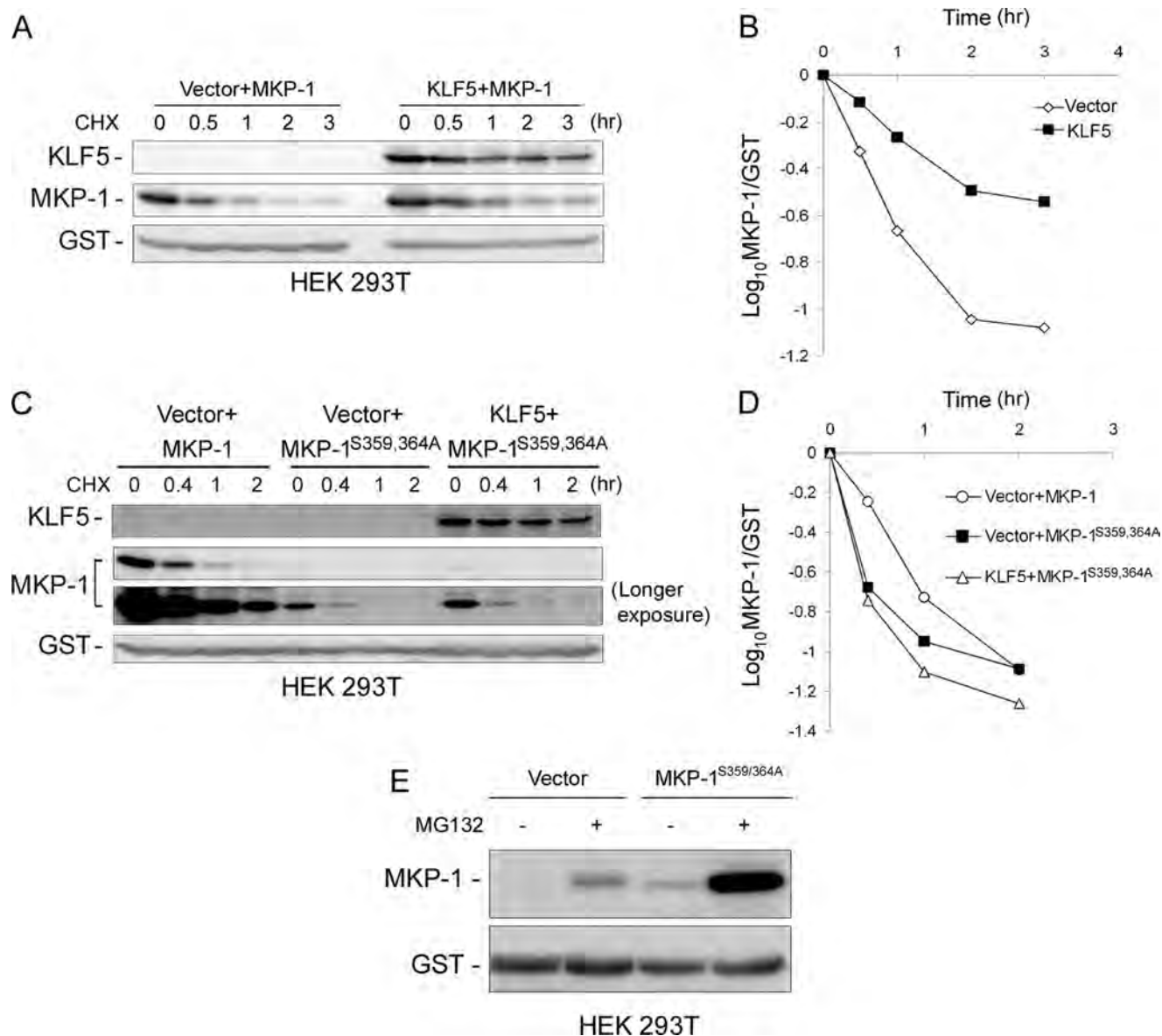


FIGURE 6. KLF5 stabilizes WT MKP-1 but not the MKP-1^{S359A/S364A} mutant. A and B, KLF5 increases the WT MKP-1 protein stability as determined by the CHX chase assay. HEK 293T cells (the endogenous KLF5 and MKP-1 expression levels are low) were transfected with MKP-1 and KLF5 expressing constructs. The CHX chase assay was performed 2 days after the transfection. The band intensities were quantified using densitometry. C and D, MKP-1^{S359A/S364A} is less stable than WT MKP-1 and KLF5 cannot stabilize MKP-1^{S359A/S364A}. E, MG132 (20 μ M, 4 h treatment) increases the endogenous MKP-1 and the exogenous MKP-1^{S359A/S364A} protein levels in HEK293T cells. GST, glutathione S-transferase.

increases the pro-survival phosphatase MKP-1 protein levels in breast cells (Figs. 1–3). Following that, we demonstrated that MKP-1 can partially rescue KLF5 knockdown-induced apoptosis in MCF10A (Fig. 2). Furthermore, we characterized the mechanism by which KLF5 up-regulates MKP-1 in breast cells and found that KLF5 decreases MKP-1 protein degradation via pERK-mediated MKP-1 phosphorylation (Figs. 4–6). Finally, we showed that KLF5 activates ERK signaling through its direct target gene *FGF-BP* (Fig. 7).

Multiple lines of evidence suggests that KLF5 up-regulates MKP-1 expression through activating the ERK signaling. First, KLF5 does not directly regulate the *MKP-1* gene at the transcriptional level (Fig. 3). Second, KLF5 increases MKP-1 protein stability (Fig. 4). Third, KLF5 maintains and increases pERK levels in multiple breast cell lines, and pERK is sufficient

and essential for KLF5-mediated MKP-1 stabilization (Figs. 2 and 5). Additionally, we confirmed that the pMKP-1^{Ser-359} is regulated by KLF5-FGF-BP-pERK signaling (Figs. 5 and 7). Furthermore, the mutation of two key pERK phosphorylation sites (S359A/S364A) in MKP-1 abrogates KLF5-mediated MKP-1 stabilization (Fig. 6). These findings strongly suggest that KLF5 up-regulates MKP-1 via pERK-mediated MKP-1 protein phosphorylation and stabilization (Fig. 7C).

As described in the Introduction, pERK can increase *MKP-1* expression at both transcriptional and post-translational levels (23–26). Indeed, we found that KLF5 increases the *MKP-1* mRNA levels in TSU-Pr1 (3). However, *MKP-1* mRNA levels are not regulated by KLF5 in tested breast cell lines. It is possible that the signaling transduction from pERK to MKP-1 transcription is inactive in breast cells. The phosphorylation mediated

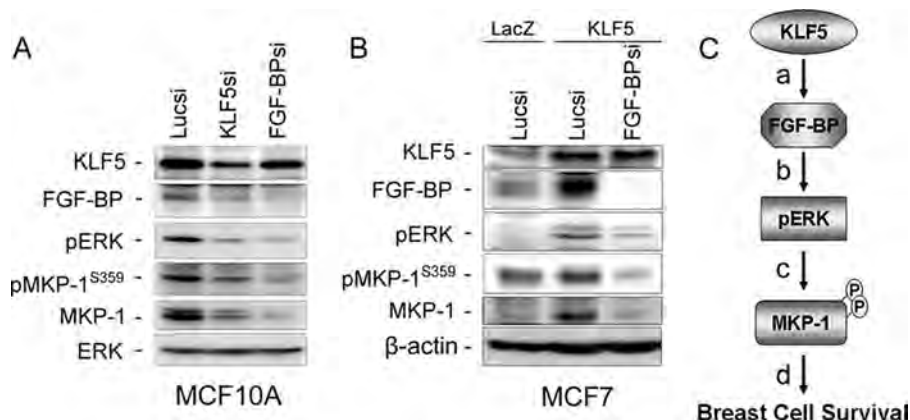


FIGURE 7. KLF5 activates ERK-MKP-1 signaling through FGF-BP in breast cells. *A*, knockdown of either KLF5 or FGF-BP down-regulates pERK, pMKP-1^{Ser-359}, and total MKP-1 protein levels in MCF10A. *B*, FGF-BP depletion blocks KLF5 overexpression induced up-regulation of the pERK, pMKP-1^{Ser-359}, and total MKP-1 protein levels in MCF7. *C*, a linear model of KLF5 promoting breast cell survival. *a*, KLF5 directly induces the *FGF-BP* transcription. *b*, FGF-BP activates the ERK signaling. *c*, the activated ERK phosphorylates MKP-1 at Ser-359 and Ser-364 and prevents its degradation. *d*, the elevated MKP-1 promotes breast cell survival.

protein stabilization is the primary mechanism by which pERK increases MKP-1 expression in breast cells.

To understand the mechanism by which KLF5 up-regulates ERK signaling, we tested the possibility that KLF5 up-regulates epidermal growth factor receptor, a known KLF5 target gene in squamous epithelial cells (34) and a well known receptor tyrosine kinase activating ERK. However, we did not detect any changes of epidermal growth factor receptor expression when KLF5 is knocked down in MCF10A (data not shown). A recent study in our laboratory indicated that KLF5 directly controls transcription of the *FGF-BP* gene in breast cells.³ FGF-BP is a secreted protein that can bind to fibroblast growth factors and promote FGF receptor-mediated ERK activation (33). We subsequently tested if FGF-BP mediates ERK activation and MKP-1 stabilization by KLF5. As expected, knockdown of either KLF5 or FGF-BP dramatically decreases the pERK, pMKP-1^{Ser-359}, and total MKP-1 levels in MCF10A (Fig. 7A). Furthermore, knockdown of FGF-BP abrogates the KLF5 overexpression induced up-regulation of pERK, pMKP-1^{Ser-359}, and total MKP-1 in MCF7 (Fig. 7B). These findings support the KLF5-FGF-BP-pERK-MKP-1 linear model proposed in Fig. 7C although it may be oversimplified under physiological conditions.

A surprising finding of this study is that MKP-1 can promote MCF10A cell survival in a phosphatase activity independent manner (Fig. 2). It has been reported that the phosphatase activity is essential for the anti-apoptotic function of MKP-1 after UV treatment in human fibroblasts (35). Because MKP-1 can de-phosphorylate multiple MAPKs, including ERK, JNK, and p38, overexpression of the catalytically inactive MKP-1^{C258S} mutant may increase multiple MAPKs at the same time. Indeed, we found that the pERK levels are dramatically increased in the MKP-1^{C258S} overexpressing MCF10A cells due to the lack of negative feedback control (Fig. 2A). Because the balance between the pro-survival pERK level and the pro-apoptosis pJNK/pp38 levels determines whether cells will live or die (16), it is possible that the elevated pERK levels contribute to the pro-survival function of MKP-1^{C258S} in MCF10A.

Besides MKP-1, KLF5 may promote breast cell survival through other downstream genes and pathways because

Besides FGF-BP, we cannot completely exclude that KLF5 up-regulates the pERK level and breast cell survival through other target genes such as *SURVIVIN* and *PIM1* and other mechanisms, such as interaction with PARP1.

In summary, we showed that KLF5 is a pro-survival transcription factor in breast cells. KLF5 functions partially through pERK-mediated MKP-1 protein phosphorylation and stabilization. Finally, we found that KLF5 may up-regulate the pERK levels through the direct target gene FGF-BP in breast cells. The KLF5-FGF-BP-pERK-MKP-1 signaling axis may provide new therapeutic targets for invasive breast cancer.

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The Fbw7 Tumor Suppressor Targets KLF5 for Ubiquitin-Mediated Degradation and Suppresses Breast Cell Proliferation

Dong Zhao, Han-Qiu Zheng, Zhongmei Zhou, and Ceshi Chen

Abstract

Fbw7 is a tumor suppressor frequently inactivated in cancers. The KLF5 transcription factor promotes breast cell proliferation and tumorigenesis through upregulating *FGF-BP*. The KLF5 protein degrades rapidly through the ubiquitin proteasome pathway. Here, we show that the Skp1-CUL1-Fbw7 E3 ubiquitin ligase complex (SCF^{Fbw7}) targets KLF5 for ubiquitin-mediated degradation in a GSK3 β -mediated KLF5 phosphorylation-dependent manner. Mutation of the critical S303 residue in the KLF5 Cdc4 phospho-degrons motif (³⁰³SPPSS) abolishes the protein interaction, ubiquitination, and degradation by Fbw7. Inactivation of endogenous Fbw7 remarkably increases the endogenous KLF5 protein abundances. Endogenous Fbw7 suppresses the *FGF-BP* gene expression and breast cell proliferation through targeting KLF5 for degradation. These findings suggest that Fbw7 inhibits breast cell proliferation at least partially through targeting KLF5 for proteolysis. This new regulatory mechanism of KLF5 degradation may result in useful diagnostic and therapeutic targets for breast cancer and other cancers. *Cancer Res*; 70(11); 4728–38. ©2010 AACR.

Introduction

The F-box and WD40 repeat domain-containing 7 (Fbw7/Cdc4) protein is a bona fide tumor suppressor inhibiting cell division and growth (1). Fbw7 is inactivated in numerous human malignancies, including breast cancer by gene mutation (2–4) and expression downregulation (5, 6). Fbw7 is an F-box protein that recruits substrates for the SCF^{Fbw7} (a complex of Skp1, CUL1, and F-box proteins) E3 ubiquitin ligase. SCF^{Fbw7} degrades several well-known oncoproteins, including MYC (7, 8), Cyclin E (4, 9), Notch (10), c-Jun (11), and mammalian target of rapamycin (12, 13). All Fbw7 substrates contain at least one conserved Cdc4 phospho-degrons (CPD) sequence (T/S)PXX(S/T/E) in which the T/S residue can be phosphorylated by GSK3 (1).

The KLF5 transcription factor has been shown to play important roles in cancer (14). Accumulated evidence suggests that KLF5 promotes fibroblast, colon, bladder, and breast cell proliferation (15–17). KLF5 is highly expressed in estrogen receptor (ER) α -negative basal-type breast cancer and is an unfavorable prognostic biomarker correlated with shorter survival for breast cancer patients (18, 19). Our previous studies suggest that KLF5 promotes breast cell proliferation

through directly upregulating the *FGF-BP* gene transcription (17). More recently, inhibition of KLF5 by small interfering RNA (siRNA) using nanoparticles has been shown to efficiently inhibit tumor growth *in vivo* (20). These findings define KLF5 as an oncogenic transcription factor and a potential therapeutic target for invasive breast cancer and other cancers.

KLF5 is an unstable protein with a short half-life (21). KLF5 can be degraded through the ubiquitin-dependent and ubiquitin-independent mechanisms (21, 22). Previously, we showed that the major KLF5 TAD contains destruction motifs (degrons) that recruit E3 ligases for KLF5 ubiquitination and degradation (21). Besides the PY (³²⁵PPSY) motif that recruits WWP1 (23), we noticed that the KLF5 TAD also contains two putative evolution-conserved CPD motifs (³⁰³SPPSS and ³²³TPPPS) that could recruit Fbw7 containing E3 ligase complex SCF^{Fbw7}. Given the significant roles of Fbw7 and KLF5 in human cancers, it is important to know whether Fbw7 promotes KLF5 degradation.

In this article, we show that Fbw7 targets KLF5 for ubiquitin-mediated proteasomal degradation. We show that the GSK3 β kinase is involved in the KLF5 S303 phosphorylation that is required for Fbw7-mediated KLF5 degradation. Importantly, we found that Fbw7 suppresses breast cell proliferation at least partially through promoting KLF5 proteolysis. These findings help us understand the regulatory mechanism of KLF5 in human cancer.

Materials and Methods

Antibodies and reagents

The rabbit polyclonal anti-KLF5 and anti-WWP1 antibodies (Ab) are kindly provided by Dr. J.T. Dong (Emory University,

Authors' Affiliation: The Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

D. Zhao and H-Q. Zheng contributed equally to this work.

Corresponding Author: Ceshi Chen, Albany Medical College, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208. Phone: 518-262-2936; Fax: 518-262-3065; E-mail: chenc@mail.amc.edu.

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Atlanta, GA). The anti- β -actin, anti-FLAG, and anti-glutathione S-transferase Abs are from Sigma. The anti-glyceraldehyde-3-phosphate dehydrogenase Ab is from Cell Signaling. The anti-MYC 9E10, anti-HA, anti-Ub, and anti-ER α Abs are from Santa Cruz Biotechnology. The anti-FGF-BP and goat anti-KLF5 Abs are from R&D Systems. The rabbit polyclonal anti-KLF5 pS303 Ab was generated using the keyhole limpet hemocyanin-conjugated peptide "FLPQQATYFPSPS(p303)PPS" (Panora Biotech). The sera were collected and affinity purified. The Ab was diluted with 1:10,000 in 3% bovine serum albumin (BSA) for Western blotting. Calf intestinal alkaline phosphatase (CIP; 20 U/ μ L) is from Promega.

Cell culture and transfection

Wild-type (WT) and Fbw7 null DLD1 cells (kindly provided by Drs. B. Vogelstein and K.W. Kinzler, Johns Hopkins University, Baltimore, MD) were cultured in McCoy's 5A supplemented with 10% fetal bovine serum (FBS). SUM149 was cultured in Ham's F-12 supplemented with 5% FBS, 5 μ g/mL insulin, and 1 μ g/mL hydrocortisone. All transient transfections for plasmids and siRNAs were performed using Lipofectamine 2000 (Invitrogen). All chemically synthesized siRNAs were purchased from Ambion and transfected at 10 nmol/L final concentration. The siRNA target sequences are provided in Supplementary Table S1.

Expression plasmids

The pcDNA3 plasmids expressing WT KLF5, Δ 323-248, Δ 299-348, and Δ 321-328 have been described in our previous study (21). The KLF5-S303A, KLF5-S307A, KLF5-T323A, and KLF5-T323A/T327A were generated using the PCR-directed mutagenesis method. Three FLAG tags were added to the COOH-terminus of KLF5 and its mutants. All FLAG- and MYC-tagged WT and mutant Fbw7 plasmids are kindly provided by Dr. B.E. Clurman (Fred Hutchinson Cancer Research Center, Seattle, WA). The *Fbw7*- γ and *Fbw7*- γ -F genes were amplified and subcloned into the pLenti6 vector.

Quantitative reverse transcription-PCR assays

Total RNA was isolated from cells using the Trizol reagent (Invitrogen) and subjected to reverse transcription with random hexanucleotide primers using the iScript cDNA Synthesis kit (Bio-Rad). Quantitative real-time PCR was performed on the ABI-7300 system, using Roche FastStart SYBR Green Master containing Rox (Roche Diagnostics). The primer sequences used in this study are listed in Supplementary Table S2.

Immunofluorescence staining

HEK293FT cells (5×10^4) were plated on a gelatin-coated glass slide. The cells were transfected with KLF5 and FLAG-tagged Fbw7 isoforms, respectively. Two days after transfection, the cells were treated with 10 μ mol/L MG132 for 4 hours and then were fixed using 4% paraformaldehyde at 4°C overnight. The slides were washed with PBS and permeabilized by 0.2% Triton X-100 in PBS for 5 minutes. The cells were then quenched with 50 mmol/L NH_4Cl for 5 minutes

and blocked with 10% goat serum for 1 hour at room temperature. The anti-KLF5 Ab (1:100) and anti-FLAG M2 mouse monoclonal Ab (1:300) were diluted with 0.1% BSA to stain the cells at 4°C overnight. The slides were washed and incubated with secondary Abs [rhodamine goat anti-rabbit Ab from Jackson ImmunoResearch (1:150) and Alexa-488 goat anti-mouse from Molecular Probes (1:150)] in 5% goat serum for 1 hour at room temperature. Fluorescent images were captured using an Olympus BX-61 microscopy at total $\times 400$ magnification.

Protein ubiquitination assays

The *in vitro* ubiquitination assay is performed using an ubiquitination kit from Enzo Life Science. The 3 \times FLAG-tagged KLF5 and KLF5-S303A substrate proteins were purified from HEK293FT cells using immunoprecipitation (IP) with FLAG M2 beads. The KLF5 proteins were eluted by using 3 \times Flag peptide (100 μ g/mL; Sigma). Similarly, the SCF^{Fbw7} E3 complexes were purified from HEK293FT cells using IP. Myc-CUL1, Rbx1, Skp1, and FLAG-Fbw7 γ -F/R338L were cotransfected. The reaction was performed with 0.75 μ L E1, 1.5 μ L E2 (Ubch5c), 0.75 μ L Mg-ATP buffer, 1.5 μ L 10 \times ubiquitination buffer, 0.75 μ L Ub, 0.75 μ L ubiquitin aldehyde, 2 μ L KLF5, 2 μ L E3 (SCF^{Fbw7} or its mutants), and H_2O in a 15 μ L volume at 30°C for 1 hour. The ubiquitinated KLF5 proteins were detected by Western blotting. The KLF5 ubiquitination assay in cells has been described in our previous study (23).

In vitro kinase assays

FLAG-tagged KLF5 and its mutants (S303A and S307A) were purified by IP. The KLF5 proteins (5 μ L) were incubated with and without 0.4 μ L active GSK3 β enzyme (New England Biolabs), 2 μ L 10 \times reaction buffer, 0.4 μ L 10 mmol/L ATP, 0.2 μ L γ -³²P-ATP (25Ci/mmol, MP Biomedicals), and H_2O up to 20 μ L. The mixtures were incubated for 1 hour at 30°C and subjected to SDS-PAGE and autoradiography.

Results

Fbw7 interacts with KLF5

To test if all Fbw7 isoforms (α , β , and γ) interact with KLF5, we cotransfected FLAG-Fbw7 and FLAG-Fbw7-F (without the intact F-box that interacts with Skp1; ref. 7) with KLF5 into HEK293FT cells. The cells were treated with the MG132 proteasome inhibitor to protect the KLF5 protein from degradation by Fbw7. We performed IP with the anti-FLAG Ab and found that all (α , β , and γ) WT and -F Fbw7 proteins interact with the KLF5 protein (Fig. 1A). These results indicate that all Fbw7 isoforms interact with KLF5 in an F-box-independent manner.

To further test if the Fbw7 WD40 repeats are responsible for KLF5 binding, we cotransfected FLAG-Fbw7-WD (8 WD40 repeats only; ref. 7) with KLF5 into HEK293FT cells and performed IP. We found that the WD40 repeats are sufficient for KLF5 binding (Fig. 1A). When the key R338 residue is mutated into L in Fbw7 γ , the protein-protein interaction is dramatically reduced (Fig. 1B). Thus, the WD40 repeats are sufficient and necessary for KLF5 binding. To test if Fbw7

binds to KLF5 CPD motifs, we mapped the KLF5 CPD motifs that are responsible for Fbw7 binding. We found that the S303A mutation in the first CPD motif dramatically decreases the protein-protein interaction whereas the T323A mutation in the second CPD motif does not (Fig. 1C). These results indicate that the KLF5 CPD motif (³⁰³SPPSS) is responsible for Fbw7 binding. Additionally, we showed that the endogenous KLF5 interacts with FLAG-Fbw7 γ -F in the RWPE1 cells (Fig. 1C).

It has been documented that Fbw7 α and γ are in the nucleoplasm and nucleolus, respectively, in U2OS cells whereas Fbw7 β is in the cytoplasm (1). To determine whether Fbw7 isoforms are colocalized with KLF5, we cotransfected FLAG-Fbw7 isoforms and KLF5 into HEK293FT cells and found that Fbw7 α and γ are localized in the nucleus whereas Fbw7 β is in the cytoplasm by immunofluorescence staining (Fig. 1D). As expected, KLF5 is predominately localized in the nucleus, although it can also be detected in the cytoplasm (Fig. 1D). The colocalization of KLF5 and Fbw7 α and γ is obvious.

Fbw7 overexpression promotes the KLF5 protein proteasomal degradation

Next, we asked whether Fbw7 overexpression decreases the KLF5 protein levels. To test this, we first cotransfected Fbw7 γ and Fbw7 γ -F with KLF5 into HEK293FT cells and found that WT Fbw7 γ dramatically reduces the KLF5 steady level compared with the empty vector and Fbw7 γ -F (Fig. 2A). MG132 can increase the KLF5 steady level in the presence of Fbw7 γ (Fig. 2A). Similar results were observed for Fbw7 α and β (Supplementary Fig. S1A and C).

To further evaluate whether Fbw7 promotes KLF5 degradation, we overexpressed WT Fbw7 γ and KLF5 into HEK293FT cells and measured the KLF5 protein half-lives by cycloheximide chase assays. We found that Fbw7 γ dramatically reduces the KLF5 half-life compared with the empty vector, Fbw7 γ -F, and Fbw7 γ -R338L (Fig. 2B). The Fbw7 γ -mediated KLF5 protein half-life decrease is completely blocked by MG132 (Fig. 2B). Similarly, Fbw7 α also significantly decreases the KLF5 protein half-life in HEK293FT cells (Supplementary Fig. S1B).

Because the KLF5-S303A loses the protein interaction with Fbw7 γ (Fig. 1C), we tested the KLF5-S303A protein degradation by Fbw7. Consequently, the KLF5-S303A protein half-life cannot be decreased by WT Fbw7 γ compared with the Fbw7 γ -F mutant in HEK293FT cells (Fig. 2C). In contrast, mutation and deletion of the other CPD motif is still sensitive to Fbw7 γ -mediated degradation (Supplementary Fig. S1E). These results clearly suggest that the CPD (³⁰³SPPSS) motif is responsible for Fbw7 γ -mediated KLF5 degradation.

The KLF5 S303 is phosphorylated by GSK3 β

KLF5-S303A cannot be recognized by Fbw7 (Fig. 1C) and cannot be degraded by Fbw7 (Fig. 2D). To further investigate whether the phosphorylation occurs at S303, we generated a KLF5-S303 phosphorylation-specific Ab using a synthesized phosphorylated peptide. This anti-KLF5 pS303 Ab works well for Western blotting as it specifically detects the phosphorylated KLF5 band from WT KLF5 and KLF5-S307A, but not KLF5-S303A (Fig. 3A). To further test whether the KLF5

phosphorylation is required for Fbw7 binding, we treated the FLAG-Fbw7 γ and KLF5-transfected HEK293FT cell lysate with different dosages of CIP and performed IP. We confirmed that the CIP treatment almost completely eliminated the KLF5 S303 phosphorylation (Fig. 3A). Importantly, the binding between FLAG-Fbw7 γ and KLF5 is significantly reduced after the CIP treatment (Fig. 3A).

It is well known that GSK3 β is the kinase for the first Ser/Thr in the CPD motifs of several Fbw7 substrates, such as MYC (1). To test if the KLF5 degradation is regulated by GSK3 β in cultured cells, we treated HeLa cells with the GSK3 inhibitor LiCl and the negative control KCl. As expected,

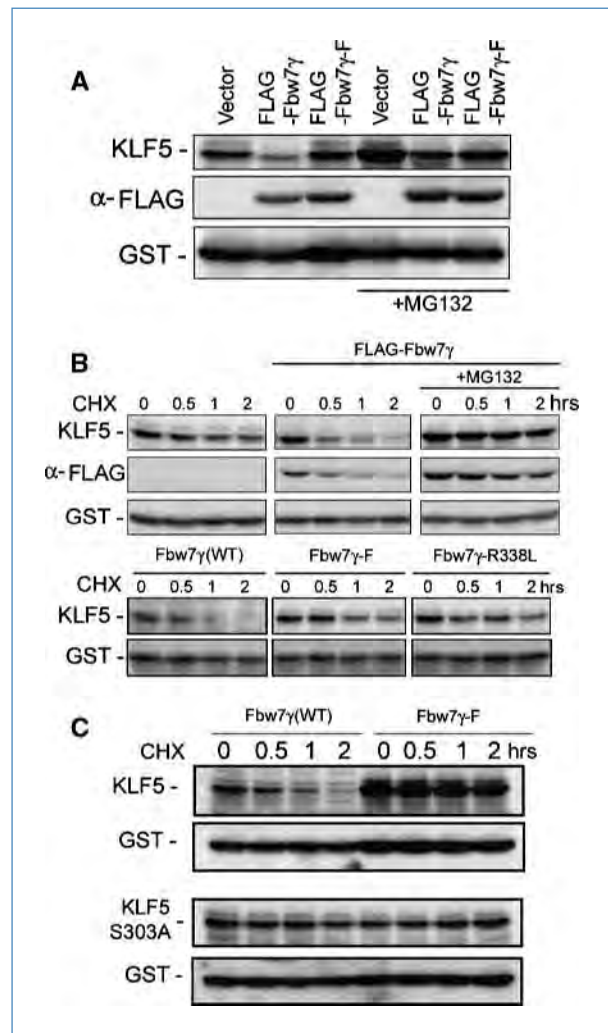
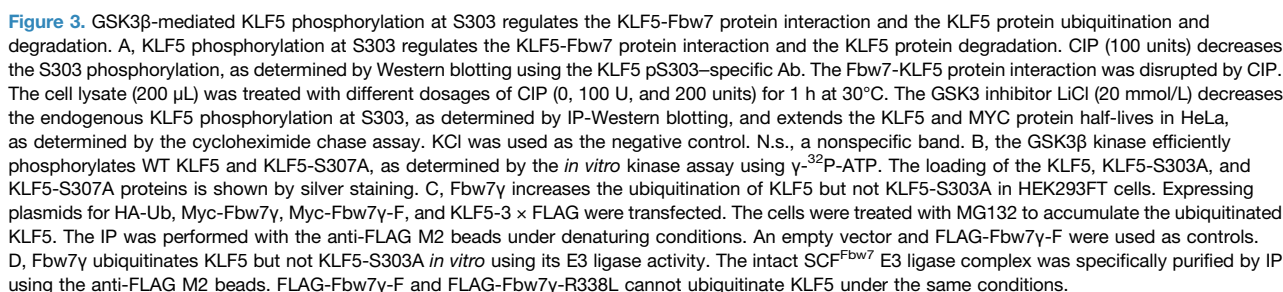


Figure 2. Fbw7 promotes proteasomal degradation of the KLF5 protein. A, FLAG-Fbw7 γ decreases the steady levels of KLF5 in HEK293FT cells, as determined by Western blotting. An empty vector and FLAG-Fbw7 γ -F were used as controls. B, FLAG-Fbw7 γ , but not FLAG-Fbw7 γ -F and FLAG-Fbw7 γ -R338L, decreases the KLF5 protein half-life in HEK293FT cells. The protein half-lives were measured by cycloheximide (CHX; 50 μ g/mL) chase assays and Western blotting. Glutathione S-transferase was used as a transfection control. The exposure times have been adjusted for each panel to compare protein degradation. C, KLF5-S303A is resistant to FLAG-Fbw7 γ -mediated degradation.



To directly test whether GSK3 β phosphorylates KLF5 at S303, we performed the *in vitro* kinase assay using the purified recombinant GSK3 β kinase and the purified recombinant KLF5/KLF5-S303A/KLF5-S307A proteins in the presence of γ -³²P-ATP. We found that GSK3 β can efficiently phosphorylate WT KLF5 and KLF5-S307A but not KLF5-S303A (Fig. 3B). These results indicate that the KLF5

To test whether Fbw7 ubiquitinates KLF5 in cultured cells, we performed the KLF5 ubiquitination assay in HEK293FT cells as described in our previous study (23). We found that WT Fbw7 γ but not the Fbw7 γ -F mutant increases the KLF5 ubiquitination (Fig. 3C). Under the same condition, KLF5-S303 cannot be efficiently ubiquitinated by Fbw7 γ (Fig. 3C). Additionally, we examined the endogenous KLF5

ubiquitination in the Fbw7 WT and knockout DLD1 colon cancer cell lines (4) and found that the endogenous KLF5 ubiquitination is decreased in Fbw7 knockout DLD1 cells (Supplementary Fig. S2A).

To test whether Fbw7 directly ubiquitinates KLF5 *in vitro*, we purified KLF5 and the SCF^{Fbw7} E3 ligase complex from HEK293FT cells. The purified FLAG-Fbw7 γ /Fbw7 γ -R338L, but not Fbw7 γ -F, complexes contain the cooverexpressed Myc-CUL1 protein (Supplementary Fig. S2B), suggesting that the intact SCF^{Fbw7} E3 complex was purified by IP Fbw7 γ , Fbw7 γ -R338L, but not Fbw7 γ -F. In the presence of the KLF5 substrate, Ub, E1, E2 (UbcH5a), and ATP, the ubiquitinated KLF5 is dramatically increased by WT Fbw7 γ , but not by Fbw7 γ -F or Fbw7 γ -R338L (Fig. 3D). Finally, we showed that Fbw7 γ cannot ubiquitinate KLF5-S303A efficiently compared with WT KLF5 *in vitro* (Fig. 3D). These results suggest that the SCF^{Fbw7} E3 ligase specifically ubiquitinates KLF5 *in vitro*.

Endogenous SCF^{Fbw7} promotes KLF5 degradation

To test if endogenous KLF5 is the substrate of endogenous Fbw7, we examined the KLF5 protein levels in the Fbw7 knockout DLD1 cells and found that KLF5 is upregulated,

like another Fbw7 substrate MYC (Fig. 4A). In the presence of MG132, there is no difference for the KLF5 protein levels between the WT and Fbw7 null cells, suggesting that WT Fbw7 targets KLF5 for proteasomal degradation. To further test whether Fbw7 promotes the KLF5 protein degradation, we compared the KLF5 protein half-lives. As shown in Fig. 4A, both the KLF5 and MYC protein half-lives are dramatically extended in the Fbw7-deficient cells compared with the WT DLD1 cells.

To further test if endogenous Fbw7 suppresses the KLF5 protein expression in other cells, we knocked down Fbw7 by two different siRNAs in HeLa, MCF10A, and BT20 cells. The Fbw7 knockdown efficiencies are about 60% to 80% in these cell lines (Fig. 4B). We could not detect the endogenous Fbw7 proteins in these cell lines (data not shown) because there are no effective anti-Fbw7 Abs for Western blotting to date (24). We found that the endogenous KLF5 and MYC protein levels are significantly elevated in all these cell lines (Fig. 4B). As expected, the *KLF5* mRNA levels are not increased by Fbw7 siRNAs (Supplementary Fig. S2C). Furthermore, knockdown of Fbw7 by two different siRNAs clearly extends both the KLF5 and MYC protein half-lives in HeLa (Fig. 4B). Consistently, the KLF5 and MYC protein half-lives

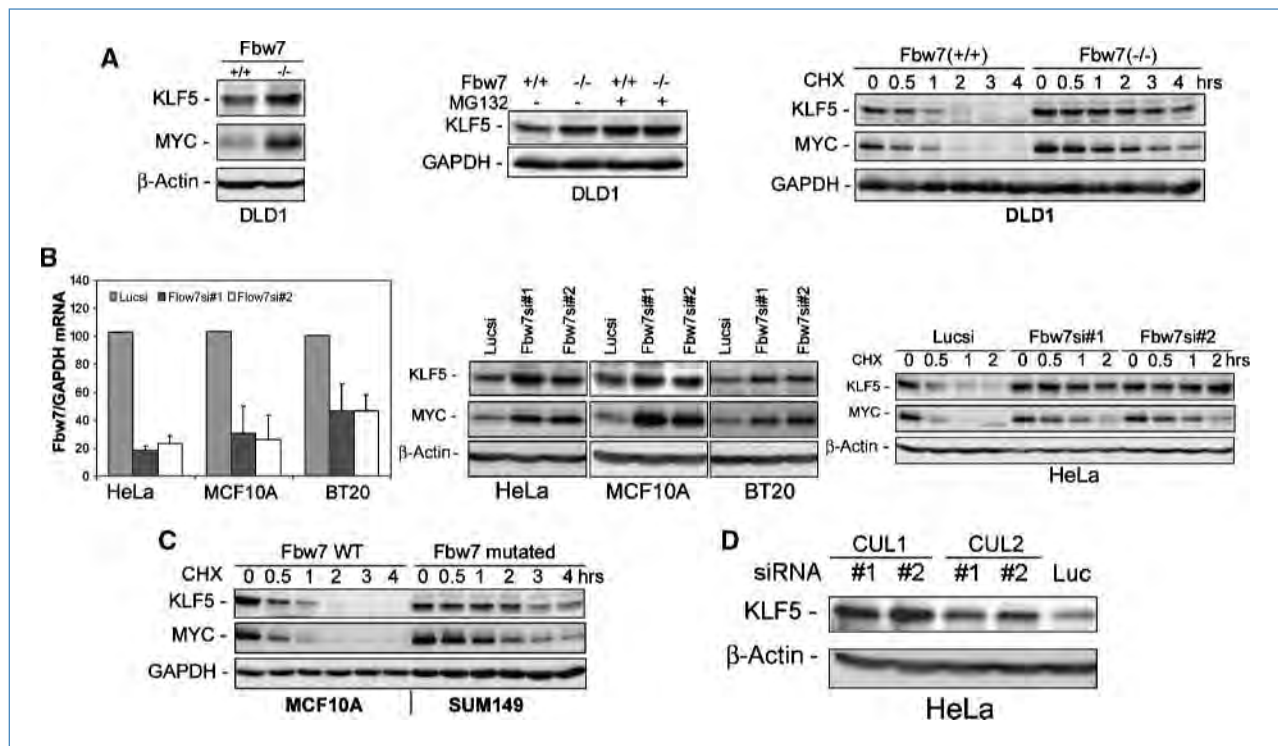


Figure 4. Inactivation of SCF^{Fbw7} increases the endogenous KLF5 protein levels through preventing KLF5 from degradation. A, endogenous KLF5 and MYC protein levels and half-lives are increased in the Fbw7 knockout cells compared with the WT DLD1 cells, as determined by the cycloheximide chase assay and Western blotting. The Fbw7-mediated endogenous KLF5 protein degradation can be blocked by MG132. B, knockdown of Fbw7 increases the endogenous protein levels of KLF5 and MYC in HeLa, MCF10A, and BT20, as determined by Western blotting. Fbw7 is knocked down by two different siRNAs, as determined by qRT-PCR. Lucsi was used as a negative control. The *KLF5* mRNA levels are not upregulated by Fbw7 siRNAs in these cells (Supplementary Fig. S2C). The protein half-lives of endogenous KLF5 and MYC are dramatically extended in Fbw7 knockdown HeLa cells, as determined by the cycloheximide chase assay. C, the protein half-lives of endogenous KLF5 and MYC are dramatically extended in Fbw7 mutated SUM149 breast cancer cells, as determined by the cycloheximide chase assay. D, depletion of *CUL1*, but not *CUL2*, by two different siRNAs increases the endogenous KLF5 protein levels in HeLa.

in the Fbw7-mutated SUM149 breast cancer cell line are much longer than those in MCF10A that has WT Fbw7 (Fig. 4C). These results strongly suggest that inactivation of endogenous Fbw7 by gene knockout, knockdown, or mutation increases the KLF5 protein stability.

Because Fbw7 functions as an adaptor for SCF^{Fbw7}, we asked whether knockdown of other SCF components, such as CUL1 (25), also increases the KLF5 protein expression. We knocked down CUL1 and CUL2 in HeLa, respectively, and found the KLF5 protein levels are specifically upregulated by knocking down CUL1 but not CUL2 (Fig. 4D). The knockdown efficiencies of CUL1 and CUL2 are ~90% as monitored by quantitative reverse transcriptase PCR (qRT-PCR; Supplementary Fig. S2D). Importantly, the *KLF5* mRNA levels are not upregulated by knocking down CUL1, suggesting that the upregulation of KLF5 occurs at the post-transcriptional level. Similar to the knockdown of Fbw7, knockdown of Rbx1 by siRNA also upregulates the KLF5 protein level in HeLa (data not shown). These findings suggest that the SCF^{Fbw7} E3 complex suppresses the KLF5 protein expression.

The expression of Fbw7 and KLF5 in breast cancer

Because the degradation of MYC by Fbw7 is isoform and cell line specific (24, 26), we asked whether the endogenous Fbw7 isoforms (α , β , and γ) also promote KLF5 degradation in a cell line-dependent manner. To test this, we knocked down Fbw7 using the isoform-specific siRNAs (27) in HeLa, MCF10A, and 184B5 cell lines. We found that knockdown of any Fbw7 isoforms (α , β , and γ) upregulates the KLF5 protein levels in HeLa with a similar extent (Fig. 5A). In the MCF10A breast cell line, knockdown of Fbw7 α and β isoforms upregulates KLF5 with a similar efficiency. However, knockdown of Fbw7 γ does not show any significant changes for KLF5 (Fig. 5A). In the 184B5 breast cell line, only knockdown of Fbw7 α clearly upregulates KLF5 (Fig. 5A). These results suggest that the degradation of KLF5 by Fbw7 is also isoform and cell line specific. Consistent with a previous report that Fbw7 α is the predominant isoform expressed in breast cancer cell lines (3), endogenous Fbw7 α appears as the major active isoform for KLF5.

Previously, we showed that KLF5 is expressed in ER α -negative breast cell lines and downregulated in ER α -positive

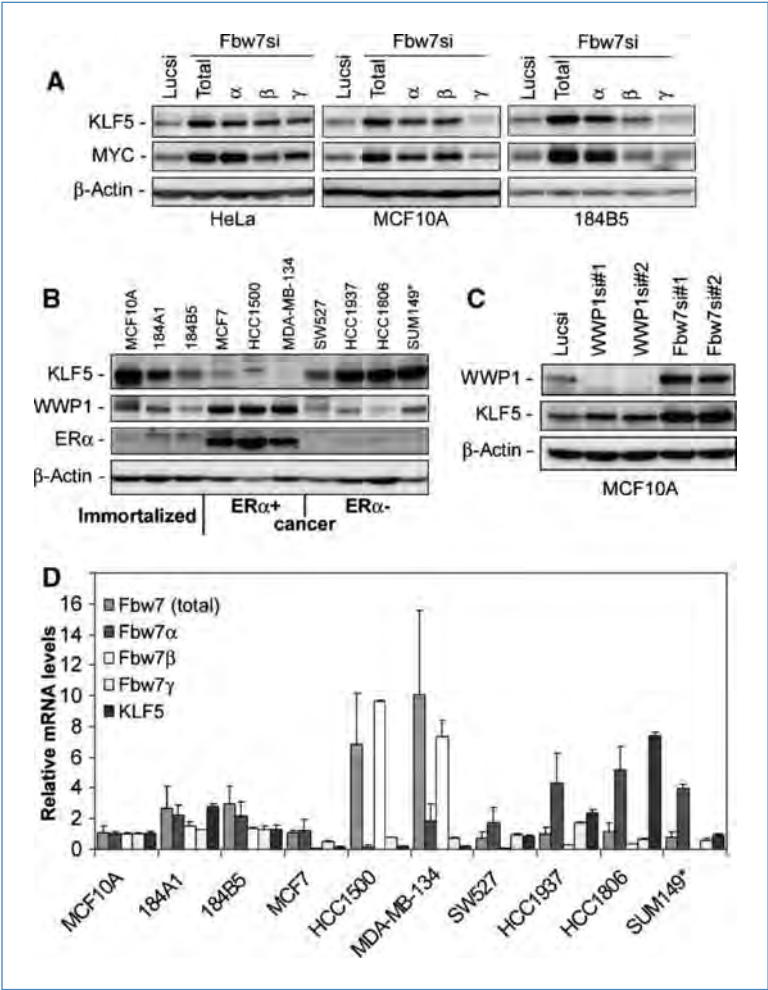


Figure 5. The expression of Fbw7 isoforms and KLF5 in breast cell lines. A, knockdown of Fbw7 by isoform-specific siRNAs differently increases the endogenous KLF5 and MYC protein levels in HeLa, MCF10A, and 184B5. B, the negative protein expression correlation between KLF5 and WWP1 in a panel of 10 breast cell lines. *, the *Fbw7* gene is inactively mutated in SUM149. C, knockdown of WWP1 and Fbw7 by two different siRNAs upregulates the endogenous KLF5 protein levels in MCF10A cells. D, the mRNA levels of *Fbw7* isoforms and *KLF5* in breast cell lines, as determined by qRT-PCR.

breast cell lines (17). The KLF5 E3 ligase WWP1 is overexpressed in ER α -positive breast cancer (28). As shown in Fig. 5B, there is a negative correlation between the KLF5 and WWP1 protein expression in a panel of 10 breast cell lines. To test whether both KLF5 E3 ligases function at the same time, we knocked down WWP1 and Fbw7 by two different siRNAs, respectively, in MCF10A and found that knockdown of Fbw7 increases the KLF5 protein levels to higher levels compared with knockdown of WWP1 (Fig. 5C). These results suggest that Fbw7 is the major E3 ligase for KLF5 in MCF10A, although both endogenous Fbw7 and WWP1 target KLF5 for degradation. Interestingly, Fbw7 and WWP1 appear to compensate each other because knockdown of either Fbw7 or WWP1 causes the expression upregulation of the other KLF5 E3 ligase (Supplementary Fig. S2E; Fig. 5C).

Finally, we measured the relative mRNA levels of *KLF5*, total *Fbw7*, and the individual *Fbw7* isoforms (α , β , and γ) in 10 breast cell lines by qRT-PCR (Fig. 5D). Among three immortalized cell lines (MCF10A, 184A1, and 184B5), the mRNA levels of *Fbw7* α and total *Fbw7* negatively correlate with the KLF5 protein levels (Fig. 5B and D). Consistent with our previous reports (17, 29), the low levels of KLF5 protein expression in ER α -positive breast cancer cells seem to be driven by the loss of *KLF5* mRNA expression. In ER α -negative cancer cell lines, the downregulation of Fbw7 is obvious compared with 184A1 and 184B5. Interestingly, inactivation of Fbw7 in SUM149 leads to the accumulation of a high level of KLF5 protein without upregulating the *KLF5* mRNA level (Fig. 5B and D).

Fbw7 suppresses the *FGF-BP* expression and breast cell proliferation through promoting KLF5 degradation

Recently, we showed that KLF5 promotes breast cell proliferation through upregulating the *FGF-BP* expression (17). To test whether Fbw7 suppresses the KLF5 transactivation function, we knocked down *Fbw7* by siRNA in MCF10A, 184B5, and MCF7 breast cells (Supplementary Fig. S3A) and found that the KLF5 protein levels are upregulated (Fig. 6A). In agreement with the fact that *FGF-BP* is one of the KLF5 transcriptional targets (17), we found that the *FGF-BP* expression upregulation occurs at the mRNA and/or protein levels (Fig. 6A). Knockdown of Fbw7 by two different short hairpin RNAs in 184B5 shows similar results (Supplementary Fig. S3B). Importantly, the depletion of KLF5 can significantly rescue the Fbw7 siRNA-induced *FGF-BP* upregulation in all three cell lines (Fig. 6A). These observations suggest that endogenous Fbw7 suppresses the KLF5 function of inducing the *FGF-BP* gene transcription in breast cells.

Because KLF5 promotes breast cell proliferation (17) and Fbw7 also targets several other oncoproteins for degradation, it is important to elucidate whether Fbw7 suppresses breast cell proliferation through KLF5. As shown in Fig. 6B, knockdown of Fbw7 significantly increases DNA synthesis in MCF10A, 184B5, and MCF7. Knockdown of Fbw7 by two different short hairpin RNAs in 184B5 also increases DNA synthesis and cell proliferation (Supplementary Fig. S3C). In agreement with our earlier report (17), depletion of KLF5 can almost completely block the Fbw7 siRNA-induced

DNA synthesis increase in all three cell lines. Additionally, depletion of KLF5 in MCF7 can rescue the Fbw7 siRNA-induced colony formation increase in soft-agar (Supplementary Fig. S3D). These results strongly argue that endogenous Fbw7 suppresses breast cell proliferation through targeting the endogenous KLF5 for degradation.

Finally, we tested whether restoring the Fbw7 expression in the SUM149 breast cancer cell line, in which the endogenous Fbw7 loses its activity by gene mutation (3), inhibits *FGF-BP* expression and DNA synthesis. FLAG-Fbw7 γ , FLAG-Fbw7 γ -F, and LacZ were overexpressed in SUM149 by lentiviruses. As expected, the KLF5 protein level, but not its mRNA level, is specifically downregulated by the WT Fbw7 γ compared with LacZ and Fbw7 γ -F (Fig. 6C). Furthermore, restoring WT Fbw7 γ in SUM149 significantly decreases the *FGF-BP* mRNA level (Fig. 6C) and DNA synthesis (Fig. 6D) compared with LacZ and Fbw7 γ -F.

Discussion

This is the first study to report that the KLF5 protein degradation is targeted by the SCF^{Fbw7} E3 ligase. We provide several lines of evidence to support that Fbw7 targets the KLF5 protein for ubiquitin-mediated proteasomal degradation and suppresses breast cell proliferation. First, Fbw7 binds to the KLF5 protein through the WD40/CPD motif interaction. Second, Fbw7 overexpression decreases the KLF5 protein level and half-life. Third, the phosphorylation of KLF5 at S303 by GSK3 β is indispensable for Fbw7 to target KLF5 for ubiquitination and degradation. Fourth, Fbw7 ubiquitinates KLF5 through its E3 ligase activity. Additionally, the inactivation of Fbw7 increases the endogenous KLF5 protein level and half-life. Finally, Fbw7 suppresses the KLF5 functions of promoting the *FGF-BP* gene expression and breast cell proliferation.

The KLF5 protein is an unstable protein with a short half-life. Previously, we reported that KLF5 degrades rapidly through the ubiquitin proteasome pathway (21). The degrons overlap with its TAD between 299 and 348. We first identified a PY motif from this region that recruits WWP1 (23). However, Fbw7 binds exclusively to the KLF5 CPD motif in a S303 phosphorylation-dependent manner (Supplementary Fig. S4). Thus, KLF5 TAD contains two different degrons that recruit Fbw7 and WWP1, respectively (Supplementary Fig. S4). Depletion of either WWP1 or Fbw7 increases the KLF5 protein levels in MCF10A; however, Fbw7 is the major E3 ligase for KLF5. Interestingly, when Fbw7 is knocked down in MCF10A, the WWP1 expression level is upregulated and vice versa (Supplementary Fig. S2E; Fig. 5C). Thus, Fbw7 and WWP1 are coordinately activated to target KLF5 for degradation.

The KLF5 protein is phosphorylated by PKC (30) and extracellular signal-regulated kinase (31). For the first time, we found that the phosphorylation of KLF5 at S303 by GSK3 β promotes the protein ubiquitination by Fbw7. Additionally, GSK3 β -mediated phosphorylation usually needs priming phosphorylation at +4 site (S307 in KLF5; ref. 1). However, KLF5-S307A can still be efficiently phosphorylated

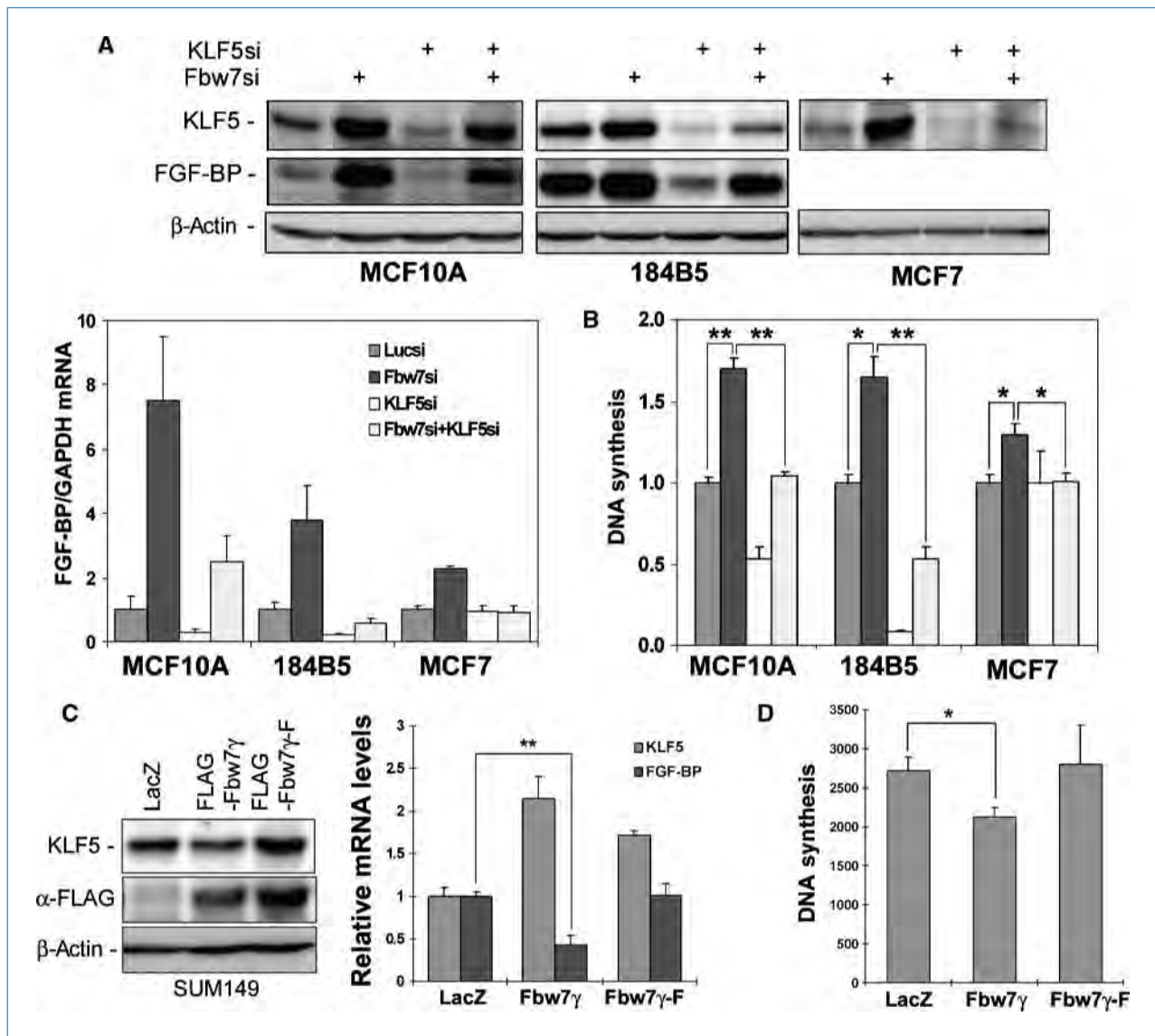


Figure 6. Fbw7 suppresses the FGF-BP expression and breast cell proliferation through promoting KLF5 degradation. **A**, knockdown of *Fbw7* by siRNA increases the FGF-BP protein and/or mRNA levels in a KLF5-dependent manner in MCF10A, 184B5, and MCF7 (the FGF-BP protein cannot be detected in MCF7 by Western blotting). The *Fbw7* and *FGF-BP* mRNA levels were determined by qRT-PCR. Knockdown of *Fbw7* by siRNA increases the KLF5 protein levels. Knockdown of KLF5 by siRNA rescues the depletion of *Fbw7*-induced FGF-BP protein and/or mRNA expression upregulation. **B**, knockdown of *Fbw7* by siRNA significantly increases the DNA synthesis in MCF10A, 184B5, and MCF7, as determined by the ^3H -thymidine incorporation. Knockdown of KLF5 by siRNA significantly rescues the depletion of *Fbw7*-induced DNA synthesis increase. **C**, the overexpression of FLAG-Fbw7 γ decreases the endogenous KLF5 protein level, but not *KLF5* mRNA level, compared with LacZ and FLAG-Fbw7 γ -F in SUM149, as determined by Western blotting and qRT-PCR. The overexpression of FLAG-Fbw7 γ significantly decreases the *FGF-BP* mRNA level. **D**, the overexpression of FLAG-Fbw7 γ significantly decreases the DNA synthesis compared with LacZ and FLAG-Fbw7 γ -F in SUM149. *, $P < 0.05$; **, $P < 0.01$ (t test).

by GSK3 β *in vitro* and in cultured cells (Fig. 3). In addition, we found that KLF5-S307A can still efficiently interact with Fbw7 γ and be ubiquitinated by Fbw7 γ (data not shown). These results suggest that the priming phosphorylation could be from other sites.

Three Fbw7 isoforms (α , β , and γ) show different subcellular localization in U2OS (1) and HEK293FT cells (Fig. 1D). KLF5 is predominately localized in the nucleus of HEK293FT cells (Fig. 1D). However, a small fraction of KLF5 has been

shown to localize in the cytoplasm (32). Nevertheless, all Fbw7 isoforms interact with KLF5 in our IP experiments after the disruption of the intact cell structures (Fig. 1A). Overexpression of any Fbw7 isoforms decrease the KLF5 protein levels (Supplementary Fig. S1A and C; Fig. 2A). Importantly, knockdown of any endogenous Fbw7 isoforms in HeLa increases the endogenous KLF5 protein levels (Fig. 5A). In breast cells, Fbw7 α seems to be the major functional endogenous isoform for KLF5.

Fbw7 has been documented to be inactivated by somatic gene mutation in a small subset (~1%) of breast cancers based on the Catalogue of Somatic Mutations in Cancer Database. Interestingly, polymorphism of the *Fbw7* gene was found to be associated with high-stage and ER α -negative breast cancers (33). *Fbw7* has been reported to be induced by the p53 tumor suppressor (6) that is frequently mutated in ER α -negative breast tumors. Indeed, the total *Fbw7* mRNA levels in ER α -negative breast cancer cell lines are generally lower than that in ER α -positive breast cancer cell lines (Fig. 5D). In addition to breast cancer, Fbw7 is more frequently mutated in tumors from the endometrium (15%), large intestine (9%), thyroid (8%), hematopoietic and lymphoid tissue (8%), pancreas (3%), and others. Consistently, the conditional knockout of *Fbw7* in the T-cell lineage of mice shows thymic hyperplasia and eventually the mice develops thymic lymphoma (34). The *Fbw7* heterozygous knockout mice increase susceptibility to radiation-induced tumorigenesis (6). It is well documented that KLF5 plays oncogenic roles in breast cancer (17), colon cancer (35), leukemia (36), and pancreatic cancer (37). These findings suggest that genetic inactivation of Fbw7 in a variety of cancers could promote cancer progression through accumulating KLF5.

Fbw7 has been suggested to be a tumor suppressor controlling the level of the key cell cycle regulatory protein Cyclin E (1). In this study, we show that Fbw7 inhibits KLF5. Importantly, KLF5 seems to be a critical substrate for Fbw7 to suppress breast cell proliferation because depletion of KLF5 can rescue the inactivation of Fbw7-induced DNA synthesis increase (Fig. 6A and B). KLF5 has been shown to

promote G₁-S and G₂-M cell cycle transition through upregulating the *FGF-BP*, *Cyclin D1*, and *Cyclin B1* protein levels (15, 16). Thus, Fbw7 suppresses cell cycle progression by directly and indirectly controlling multiple cell cycle regulatory proteins.

In summary, we show that Fbw7 targets KLF5 proteins for ubiquitin-mediated proteasomal degradation in a S303 phosphorylation-dependent manner. We show that Fbw7 suppresses KLF5's functions of promoting gene transcription and breast cell proliferation. Given the frequent inactivation of Fbw7 in breast and other cancers, these findings may help us further understand the roles of Fbw7 and KLF5 in cancer development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Essential role of KLF5 transcription factor in cell proliferation and differentiation and its implications for human diseases

Jin-Tang Dong · Ceshi Chen

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Abstract KLF5 (Kruppel-like factor 5) is a basic transcription factor binding to GC boxes at a number of gene promoters and regulating their transcription. KLF5 is expressed during development and, in adults, with higher levels in proliferating epithelial cells. The expression and activity of KLF5 are regulated by multiple signaling pathways, including Ras/MAPK, PKC, and TGF β , and various posttranslational modifications, including phosphorylation, acetylation, ubiquitination, and sumoylation. Consistently, KLF5 mediates the signaling functions in cell proliferation, cell cycle, apoptosis, migration, differentiation, and stemness by regulating gene expression in response to environment stimuli. The expression of KLF5 is frequently abnormal in human cancers and in cardiovascular disease-associated vascular smooth muscle cells (VSMCs). Due to its significant functions in cell proliferation, survival, and differentiation, KLF5 could be a potential diagnostic biomarker and therapeutic target for cancer and cardiovascular diseases.

Keywords KLF5 · Proliferation · Survival · Differentiation · Homeostasis · Tumorigenesis · Cardiovascular diseases

Introduction

Transcription factors regulate diverse cellular processes, including proliferation, cell cycle, apoptosis, migration, and differentiation, by controlling gene expression. Accumulating evidence suggests that genetic aberrations of SP/KLF (Kruppel-like factor) transcription factors are involved in the development of various human diseases, including cancer and cardiovascular diseases, as reviewed previously [1–7]. The KLF family consists of ~20 members in humans, and is structurally characterized by three tandem zinc-finger domains at the C-terminus. Several members of the KLF family, such as KLF2 [8], KLF4 [9, 10], KLF5 [11], KLF6 [12, 13], and KLF8 [14], have been demonstrated to play vital roles in the development of various human cancers.

KLF5, also named BTEB2 [15] and IKLF, belongs to the KLF family. KLF5 is widely expressed at varying levels in different tissues. As a basic transcription factor, KLF5 regulates a number of important target genes, such as *cyclin D1*, *cyclin B*, *PDGF α* , and *FGF-BP*. KLF5 has essential roles in cell cycle regulation, apoptosis, migration, and differentiation. In recent years, the study of KLF5 has been dramatically expanded. This review article comprehensively summarizes the biochemical and molecular aspects of KLF5, including its gene and protein structures, expression patterns, protein posttranslational modifications, interacting proteins, downstream target genes, and upstream regulators. Following that, we review the functions of KLF5 in various physiological and pathological cellular processes, including cell proliferation, survival, migration, differentiation, and stemness. Additionally, we outline all in vivo studies of KLF5 transgenic mouse models. Finally, the relationships of KLF5 and human diseases are summarized and future research directions for KLF5 are proposed.

J.-T. Dong (✉)
Department of Hematology and Medical Oncology,
Department of Urology and Winship Cancer Institute,
Emory University School of Medicine, 1365-C Clifton Road,
Atlanta, GA 30322, USA
e-mail: jdong2@emory.edu

C. Chen
The Center for Cell Biology and Cancer Research, Albany
Medical College, 47 New Scotland Ave., Albany,
NY 12208, USA
e-mail: chenc@mail.amc.edu

Biochemistry

The gene and protein structures

The *KLF5* gene is located at 13q21, spanning ~18.5 kb genomic DNA with four exons. The full-length cDNA of human *KLF5* consists of 3,350 bp with a 324-bp 5'-untranslated region (UTR), a 1,652-bp 3'-UTR, and a 1,374-bp sequence coding for a 457 amino acid polypeptide (Fig. 1). Similar to other KLFs, the C-terminus of KLF5 protein contains three zinc-finger (ZF) domains, which function in DNA binding. KLF5 has a proline rich transactivation domain (TAD) before the ZF domains [15, 16].

Expression

KLF5 is widely expressed at varying levels in different tissues. Based on Northern blot analysis, high levels of *KLF5* mRNA are present in the human and mouse digestive tract including intestine, colon, and stomach, and pancreas, placenta, testis, prostate, skeleton muscle, and lung [15, 17, 18]. *KLF5* mRNA was also detected in human and rabbit bladder and uterus [19, 20]. Although the expression of KLF5 appears mostly to be epithelial, KLF5 is also expressed in cardiovascular SMCs [19], cornea [21], lymphoid cells [22], and neuronal cells [23]. While most tissues express a 3.3-kb transcript of *KLF5*, that expressed in the testis is about 1.5 kb [18].

Accumulated evidence suggests that KLF5 is more highly expressed in proliferating cells than in differentiated cells [17]. For example, *KLF5* shows temporal changes in expression during embryogenesis [17, 24, 25]. During

mouse development, *KLF5* mRNA continues to accumulate at a high rate in the basal layer of the epidermis and in the base of the intestinal crypts [24]. Consistently, the KLF5 protein is also exclusively expressed in proliferating epithelial cells at the base of the crypts of the intestine but not in the terminally differentiated epithelial cells in the villi [11].

The KLF5 protein is primarily expressed in the nucleus [18]. However, KLF5 also contains a nuclear export signal (NES) located next to a sumoylation site (Fig. 1) [26]. Du et al. [26] found that sumoylation facilitates KLF5 nuclear localization by inactivating NES.

Posttranslational modifications

KLF5 proteins undergo different posttranslational modifications that modulate the protein level or transactivation activities of KLF5. Such modifications include phosphorylation, acetylation, ubiquitination, and sumoylation (Fig. 1). While KLF5 phosphorylation positively regulates its activity and KLF5 ubiquitination negatively regulates its protein level, the function of acetylation and sumoylation is context-dependent.

KLF5 phosphorylation by PKC at S153 increases the transactivation activities of KLF5 [27]. Zhang et al. [27] reported that a point mutation (S153A) reduces its transactivation function, and that phosphorylation of KLF5 enhances its interaction with CREB-binding protein (CBP). Whether KLF5 is also phosphorylated at other sites by other kinases is still unclear.

KLF5 has been documented to be acetylated by p300 and deacetylated by HDAC1 and SET [28, 29]. Miyamoto et al. [28] reported that the acetyl transferase, p300,

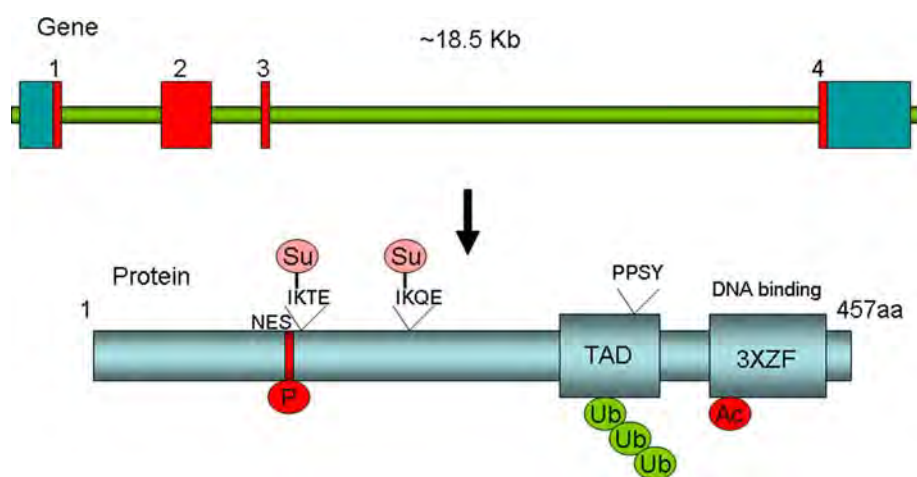


Fig. 1 The human *KLF5* gene and protein structures. The human *KLF5* genome contains four exons (exon 1, 585 bp; intron 1, 2,272 bp; exon 2, 874 bp; intron 2, 1,089 bp; exon 3, 60 bp; intron 3, 11,824 bp; and exon 4, 1,831 bp). The KLF5 protein contains three zinc-finger (ZF) domains, one major transactivation domain (TAD)

with a PY motif (PPSY328), and a nucleus export signal (NES). The KLF5 protein undergoes different types of posttranslational modifications, including phosphorylation (P at S153), acetylation (Ac at K369), ubiquitination (Ub), and sumoylation (Su at K162 and K209)

acetylates KLF5 at K369, which appears to enhance the transactivation activity of KLF5. The notion was fully confirmed by an independent study: using a KLF5 K369 acetylation specific antibody, Guo et al. [30] demonstrated that TGF β recruits p300 to acetylate KLF5. The SET histone chaperone was shown to negatively regulate the function of KLF5 in DNA binding and cell proliferation, which is accompanied by an inhibition of KLF5 acetylation [28]. The deacetylase HDAC1 can also interact with KLF5 to inhibit the binding of KLF5 to DNA as well as KLF5-mediated promoter activation through inhibiting the KLF5 acetylation by p300 [29].

KLF5 has been shown to be ubiquitinated. Chen et al. [31] found that KLF5 is degraded through the ubiquitin-proteasome pathway in epithelial cells. Like other crucial transcription factors, such as p53 and c-MYC, KLF5 turns over rapidly. The KLF5 protein half-life is ~ 1.5 h by pulse chase assays. The destruction domain is within TAD of KLF5. Further investigation revealed that the WWP1 E3 ubiquitin ligase can bind to the PY motif of KLF5 in TAD (Fig. 1), to ubiquitinate and degrade KLF5 [32]. Interestingly, the *WWP1* gene is frequently amplified and overexpressed in both breast and prostate cancers [32–34]. The WWP1 protein is highly expressed in ER α positive breast cancer while KLF5 appears to be expressed in ER α negative breast cancer [35, 36]. Our unpublished results suggest that KLF5 may also be ubiquitinated by other E3 ligases. Additionally, degradation of KLF5 by the proteasome pathway can be ubiquitin-independent [37], although the molecular mechanism is not fully understood.

KLF5 has been demonstrated to be sumoylated in two recent studies [26, 38]. Du et al. [39] applied a yeast two-hybrid screen to identify proteins that interact with KLF5 and identified the sumoylation E3 ligase PIAS1 as the potential KLF5-interacting protein. Du et al. [26] further demonstrated that mouse KLF5 is sumoylated at lysine residues 151 and 202, and that sumoylation facilitates nuclear localization and function of KLF5 by inactivating

NES located next to K151 (Fig. 1). The sumoylation of human KLF5 was reported in an independent study [38].

KLF5 posttranslational modifications, including sumoylation and acetylation, can switch the function of KLF5. Although KLF5 has no transcription repressor domain, Oishi et al. [38] found that KLF5 directly inhibits transcription of lipid metabolism genes, including *Cpt1b*, *Ucp2*, and *Ucp3* by recruiting co-repressors. After KLF5 is sumoylated upon agonist stimulation of PPAR δ , KLF5 recruits co-activators to induce the *Cpt1b*, *Ucp2*, and *Ucp3* transcription [38]. Similarly, KLF5 normally suppresses *p15* gene transcription. After KLF5 is acetylated upon TGF β stimulation, KLF5 increases the *p15* gene transcription [30]. Thus, KLF5 can regulate the expression of the same set of target genes toward opposite directions in response to environment stimuli.

Transcriptional target genes

KLF5 has been demonstrated to regulate many genes involved in cell proliferation, cell cycle, survival, migration, angiogenesis, stemness, and differentiation (Table 1) in different contexts. Most KLF5 target gene proximal promoters contain one or more GC rich sites [15, 16, 27]. Although KLF5 has been shown to bind to Sp1 sites, GC boxes, and CACCC boxes, there are no strictly conserved consensus core sequences [15, 16, 27].

Two genome wide microarray experiments have been performed to systematically identify KLF5-regulated genes. Chen et al. [40] stably expressed KLF5 in the TSU-Pr1 bladder cancer cell line, and performed a microarray analysis. At least 58 genes are differentially expressed between KLF5-positive and KLF5-negative TSU-Pr1 cells [40]. Many of the genes have been validated by different approaches for their differential expression, including *HBP17/FGF-BP*, *B94/TGFAIP2*, *DUSP1MKP-1*, *ADRB2*, *BCAR3*, *CD24*, *Dri42*, *DUSP5*, *EEF1A2*, *EMP1*, *EXT1*, *ITGA6*, *Lipocortin III*, *MN1*, *p27*, *PIG12*, *RAIG1*, *SAS*, *Slit*,

Table 1 Functional classification of direct KLF5 target genes

Functions	Target genes	References
Cell cycle	<i>Cyclin D1</i> , <i>Cyclin B1</i> , <i>Cdc2</i> , <i>p15</i> , <i>p27</i>	[30, 40, 49, 75, 79, 128]
Angiogenesis	<i>PDGFα</i> , <i>VEGFα</i> , <i>FGF-BP</i>	[40, 41, 55, 59, 129]
Inflammation	<i>MCP-1</i> , <i>NF-κB</i>	[56, 124]
Apoptosis	<i>Survivin</i> , <i>Pim1</i>	[45, 80]
Migration	<i>ILK</i> , <i>MMP9</i>	[82, 100]
Stemness	<i>Nanog</i> , <i>Tcl1</i> , <i>Oct3/4</i> , <i>Esrrb</i> , <i>Fbxo15</i>	[90, 92]
Differentiation	<i>SMemb/NMHC-B</i> , <i>SM22α</i> , <i>PAI-1</i> , <i>Egr-1</i> , <i>PPARγ</i> , <i>iNOS</i>	[19, 46, 63, 117, 118, 130]
Fatty acid metabolism	<i>Cpt1b</i> , <i>Ucp2</i> , <i>Ucp3</i> , <i>FASN</i>	[38, 47]
Others	<i>Lactoferrin</i> , <i>TCR Dβ1</i> , <i>γ-globin</i> , <i>MAO-A/B</i> , <i>KLF4</i> , <i>Lama1</i> , <i>DAF</i> , <i>EGFR</i>	[18, 22, 76, 131–137]

TGF α , *TGM2*, *TIMP2*, *TMOD*, and *Wnt7a*. Wan et al. [41] generated a lung-specific Klf5 knockout mouse model and performed a microarray analysis. KLF5 regulates the expression of hundreds of genes associated with cell cycle, angiogenesis, lipid metabolism, and several paracrine signaling pathways (*PDGF-FGF*, *VEGF*, *TGF β* , and *BMP*) [41]. Interestingly, the fibroblast growth factor binding protein 1 (*FGF-BP/HBP17*) was identified as *KLF5* target gene in both studies, suggesting that FGF-BP is regulated by KLF5 in vitro and in vivo. Our unpublished results suggest that KLF5 functions through FGF-BP to promote breast epithelial cell proliferation.

Interacting proteins

To date, about 20 KLF5 interacting proteins have been identified through yeast two-hybrid screening, mass spectrometry, and co-immunoprecipitation (Table 2). Besides the KLF5 posttranslational modifiers described above, KLF5 has been shown to assemble a transcriptional complex at chromosome to regulate gene expression by interacting with multiple proteins of the transcriptional machinery. KLF5 interacts with components of the transcriptional machinery including initiation factors, TFIIB, TFIIE β , and TFIIIF β as well as the TATA box-binding protein (TBP) [16]. Recently, KLF5 has been demonstrated to bind to PPAR δ and co-suppressors NcoR and SMRT at the promoters of *Ucp2/3* and *Cpt1b* [38]. KLF5 can also mediate the modification of histone to regulate chromatin in gene regulation. Munemasa et al. [42] recently found that KLF5 recruits a novel histone chaperone named acidic nuclear phosphoprotein 32B (ANP32B) onto gene promoter, where ANP32B incorporates into promoter-region specific histone to inhibit histone acetylation and represses the transcription of a KLF5-downstream gene.

The KLF5 protein has been shown to associate with numerous transcription factors, such as retinoic acid receptor (RAR/RXR), NF κ B, p53, C/EBP β/δ , and sterol-regulatory-element-binding protein-1 (SREBP-1), to regulate gene transcription. Fujii et al. [43] reported that KLF5 forms transcriptional complexes with RAR/RXR heterodimer on *PDGF α* promoter. In a yeast one-hybrid screen using a keratinocyte-specific NF- κ B binding site as a bait, Sur et al. [44] identified KLF5 as a factor cooperating with NF- κ B in

epidermal epithelial cells. Zhu et al. [45] found that KLF5 interacts with p53 at the promoter of survivin gene in leukemia. Similarly, KLF5 was shown to interact with C/EBP β/δ at the PPAR γ promoter in adipocyte cells [46]. Most recently, transcriptional regulation of fatty acid synthase (FASN) involves an interaction between KLF5 and SREBP-1 [47]. As an essential effector for the TGF β pathway, KLF5 has been shown to interact with Smad2/3, Smad4, Myc, Miz-1, and p300 to regulate the expression of p15 [140].

Besides transcription factors, KLF5 also specifically interacts with poly(ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme important in DNA repair and apoptosis [48]. The protein interaction occurs at the proteolytic fragment of PARP-1. It was believed that the interaction has a pro-survival function through an unknown mechanism [48].

Signaling pathways

KLF5 plays important roles in multiple important growth factor signaling pathways, including Ras-mitogen-activated protein kinase (MAPK), PKC, TGF β , TNF α , retinoid, and androgen (Fig. 2). As a basic transcription factor, KLF5 could mediate the function of different signaling pathways in different biological processes, including cell proliferation and differentiation, which often involves transcriptional regulation of KLF5.

First, the notion that KLF5 is induced by the Ras-MAPK signaling pathway is supported by several lines of investigation (Fig. 2). In oncogenic H-Ras-transformed NIH3T3 cells, KLF5 is upregulated at both RNA and protein levels that can be blocked by inhibiting MEK or Egr-1 [49]. In SMCs, *KLF5* mRNA is rapidly and persistently induced by phorbol 12-myristate 13-acetate (PMA) through Egr-1 [50]. Nandan et al. [51] found that induction of K-Ras (V12G) results in increased expression of KLF5 in IEC-6 intestinal epithelial cells. Consistently, higher levels of KLF5 are expressed in cell lines and primary tumors from human colorectal cancer with mutated K-Ras, and primary colorectal cancers induced by the K-Ras (V12G) mutation. Additionally, KLF5 was found to be upregulated by the NEU/ErbB2 oncogene, which encodes a receptor tyrosine kinase and actions upstream of Ras [52]. Fetal bovine serum, androgen, and fibroblast growth factor have been

Table 2 KLF5 interacting proteins

Function	KLF5 interacting proteins	References
Posttranslational modifiers	WWP1, PKC, p300, HDAC1, SET, PIAS	[27–30, 32, 39]
Basic transcription factors and co-activators	TFIIB, TFIIE β , TFIIIF β , TBP, CBP, p300, ANP32B, NcoR, SMRT	[16, 27, 38, 42]
Co-transcription factors	RAR/RXR, NF κ B, p53, C/EBP β/δ , SREBP-1, PPAR δ	[38, 44–47, 59, 129]
Others	PARP-1	[48]

Deletion analyses of the promoter region of rat *KLF5* suggest that at least three regions are important for *KLF5* transcription, including the GC box, CCAAT box, and NF-1 binding site. Gel mobility shift assays (EMSAs) demonstrated that factors Sp1, CBFa, and NF-1 bind to the *KLF5* promoter [72]. A Sp1 site is essential for a basal human *KLF5* promoter activity, and the binding of Sp1 to this element has been confirmed by EMSA [54]. An Egr-1 binding site at human *KLF5* promoter is essential for the

KLF5 induction by PMA [50]. Recently, Bialkowska et al. [73] screened compounds that regulate the 1,959 bp *KLF5* promoter and found that phosphoinositide 3-kinase (PI3K) inhibitors (LY294002 and Wortmannin) and that receptor tyrosine kinase (RTK) inhibitors (PDGFR inhibitor AG17 and ErbB2 inhibitor AG879) suppresses the *KLF5* transcription in colon cancer cell lines (Fig. 2). Interestingly, AG17 appears to reduce the *KLF5* expression through inhibiting the EGR-1 protein expression in colon cancer cell lines [73].

Cellular and physiological function

Cell cycle and proliferation

At present, the best known function of KLF5 is its stimulatory role in the proliferation of different types of cells, including fibroblasts, epithelial cells, and SMCs. Sun et al. found that ectopic expression of KLF5 into NIH3T3 cells significantly increased the rate of cell proliferation and caused a phenotype of transformation in a soft-agar assay [53]. As described above, KLF5 is upregulated in oncogenic H-Ras-transformed NIH3T3 cells [49]. Inhibition of KLF5 expression with KLF5-specific small interfering RNA (siRNA) leads to a decreased rate of proliferation and a significant reduction in colony formation. Similarly, Nandan et al. [51] found that K-Ras(V12G) also induces KLF5 to promote the IEC-6 intestinal epithelial cell proliferation. These findings indicate that elevated KLF5 expression is responsible for the pro-proliferative and transforming activities of oncogenic Ras [49]. Additionally, LPA stimulates cell proliferation through inducing the KLF5 expression in colon cancer cell lines [62].

Retinoids are known inhibitors of epithelial cell proliferation. In the intestinal epithelial cell line IEC-6, treatment with all-trans retinoid acid (ATRA) inhibits cell proliferation due to G1 cell cycle arrest. It was noted that this inhibition is correlated with a decrease in the levels of *KLF5* mRNA [74]. On the other hand, stable overexpression of KLF5 in IEC-6 cells abrogates the growth inhibitory effect of ATRA [74]. Furthermore, ATRA appears to inhibit cell proliferation only in human colon cancer cell lines that express a higher level of KLF5 but not in those with low levels of KLF5. These studies suggest that KLF5 is involved in the inhibitory effect of ATRA on intestinal epithelial cell proliferation [74].

KLF5 promotes cell proliferation through accelerating the G1/S and G2/M cell cycle progression. Chen et al. [40] reported that overexpression of KLF5 in the bladder cancer cell line TSU-Pr1 promotes the G1/S cell cycle progression and tumorigenesis. Nandan et al. [51] demonstrated that KLF5 also promotes the G2/M transition in Ras

transformed fibroblasts. In addition to inducing cyclin D1 and inhibiting p27 and p15 [40], KLF5 also upregulates cyclin B1 and Cdc2 [75]. In mouse primary cultures of esophageal keratinocytes, KLF5 increases cell proliferation through upregulating EGFR and the MEK/ERK signaling [76].

In contrast, KLF5 has also been found to inhibit cell proliferation in several cancer cell lines in vitro. For example, in the TE2 esophageal cancer cell line, stable expression of KLF5 inhibits cell proliferation [77]. Chen et al. [78] reported that KLF5 inhibits colony formation in the DU145 and 22Rv1 prostate cancer cell lines. KLF5 has shown a growth promoting role in the IEC-18 and IMCE immortalized intestinal cell line but a growth inhibitory role in several colon cancer cell lines [79]. In a recent study by Guo et al. [30], KLF5 was identified as a TGF β cofactor, and when TGF β was present, KLF5 plays an inhibitory role in cell proliferation. The inhibitory effect of KLF5 on the proliferation of cancer cells also appears to be TGF β -dependent [30]. Based on these observations, KLF5 regulates cell proliferation in a context-dependent manner.

Apoptosis

KLF5 has an anti-apoptosis function. In leukemia, Zhu et al. [45] found that KLF5 induces the expression of survivin, a survival factor making cells resistant to apoptosis. Down-regulation of KLF5 by siRNA decreases the expression of survivin and thus makes cells prone to doxorubicin-induced apoptosis. Mechanistically, KLF5 binds to the promoter of survivin gene and interacts with p53 to abrogate p53-repressed survivin expression [45]. KLF5 can also modulate apoptosis in a p53-independent manner [80]. When KLF5 is knocked down by RNAi, cells become more sensitive to 5-fluorouracil induced apoptosis, regardless of p53 status. KLF5 siRNA-induced apoptosis is associated with reduced BAD phosphorylation and with downregulation of PIM1, and transfection of wild-type (WT) Pim1 is sufficient to rescue the phenotype [80]. KLF5 can also make SMCs resistant to apoptosis. Suzuki et al. found that KLF5 confers apoptotic resistance in vascular lesions through interacting with PARP-1, a nuclear enzyme important in DNA repair and apoptosis. Acetylation of KLF5 under apoptotic conditions increases the interaction, and the acetylation-deficient mutant of KLF5 loses the capability to inhibit apoptosis [48]. Our recent results suggest that KLF5 can promote breast epithelial cell survival by increasing the MKP-1 protein levels [81].

Migration

KLF5 promotes the migration of epithelial cells, which is an important process in normal epithelial homeostasis in

the gut and skin [82]. The mechanism involves the induction of integrin-linked kinase (ILK), which in turn activates Cdc42 and myosin light chain to regulate cell migration and motility [82]. When KLF5 is stably expressed in TSU-Pr1 [40], cell migration is also increased, as shown by a scratch assay (unpublished data).

Differentiation

Based on a large number of published papers, it is becoming evident that KLF5 regulates the differentiation of epithelial cells, SMCs, and adipocytes.

KLF5 plays an essential role in epithelial differentiation and homeostasis. During epithelial homeostasis, stem cells divide to produce progenitor cells, which further proliferate to generate the cell mass for mature epithelia [83]. KLF5 is highly expressed in proliferating epithelial cells such as immortal but untransformed epithelial cell lines and proliferating primary cultures of epithelial cells, which mostly represent progenitor cells [74, 78, 79, 84]. In normal intestine, KLF5 is expressed at a higher level in basal rapidly proliferating cells, but at a lower level in mature and differentiated cells [59], and knockout of one KLF5 allele significantly reduced the size of villi in mouse intestine [59]. In another *in vivo* study, overexpression of KLF5 in epidermis caused hyperplasia of basal cells but lack of mature skin [85]. In the HaCaT epidermal epithelial cell line treated with TGF β , which represents a model of TGF β -induced epithelial differentiation, KLF5 plays an essential role [30].

Although KLF5 is associated with the proliferative phenotype of SMCs, it is induced in activated SMCs to modulate the state of differentiation in response to injury and contribute to vascular regeneration [86–88]. KLF5 upregulates the expression of genes involved in the SMC differentiation, including SMemb/NMHC-B [19] and SM22 α [63].

KLF5 is also necessary for adipocyte cell differentiation. In 3T3-L1 pre-adipocyte cells, which differentiate in the presence of micro-molar arsenic, arsenite treatment induces the expression of KLF5 [89]. A role of KLF5 in adipose differentiation was further demonstrated in a mouse model, where neonatal heterozygous KLF5 knockout mice exhibit a marked deficiency in white adipose tissue development [46]. In 3T3-L1 preadipocytes, KLF5 expression is induced at an early stage of differentiation, which is followed by the expression of PPAR γ 2. Constitutive overexpression of dominant-negative KLF5 inhibits adipocyte differentiation, whereas overexpression of WT KLF5 induces differentiation even without hormonal stimulation. Embryonic fibroblasts from KLF5 (\pm) mice also have an attenuated adipocyte differentiation [46]. Mechanistically, it appears that KLF5 expression is

induced by C/EBP β and δ , and, in turn, KLF5 acts in concert with C/EBP β/δ to activate the PPAR γ 2 expression [46].

Stemness

Accumulated evidence suggests that KLF5 may be essential for self-renewal of embryonic stem cells (ESCs). Jiang et al. [90] reported that Klf5, Klf4, and Klf2 redundantly maintain mouse ESC self-renewal and pluripotency through regulating the *Nanog*, *Tcl1*, *Esrrb*, and *Fbxo15* expression, and Klf4 and Klf5 showed the same pattern of expression changes during the differentiation of embryonic stem cells [91]. Indeed, Parisi et al. [92] not only confirmed that Klf5 plays an important role in self-renewal of mouse ESCs but also found that Klf5 knockdown alone causes differentiation of ESCs. Furthermore, constitutive expression of Klf5 attenuates the differentiation of ESCs, likely through direct regulation of genes involved in stem cell renewal including *Nanog* and *Oct3/4* [92]. Consistently, Ema et al. [93] demonstrated that homozygous disruption of Klf5 results in the failure of ESC and early embryonic lethality due to an implantation defect. Klf5 null ESCs show increased expression of several differentiation marker genes and frequent, spontaneous differentiation [93]. Conversely, overexpression of Klf5 in ESCs suppressed the expression of differentiation marker genes and maintained pluripotency in the absence of extrinsic factor LIF [93]. Recently a combination of four transcription factors including KLF4, Oct4, Sox2, and c-Myc was shown to reverse differentiated cells to a pluripotent state [94–96]. Klf5 can replace Klf4 to generate induced pluripotent stem (iPS) cells from differentiated MEF cells in spite of lower efficiency [97].

However, KLF5 appears to be expressed at a lower level in adult stem cells *in vivo*. A study comparing global gene expression patterns between isolated hair follicle stem cells and non-bulge basal keratinocytes showed that KLF5 is expressed at a lower level in stem cells [98]. Consistently, transgenic expression of Tcf3, a component of the Wnt signaling pathway, represses differentiation of epidermal cells by repressing the expression of KLF5 and other genes and inducing the expression of genes that are associated with an undifferentiated state and shared by embryonic and postnatal stem cells [99].

KLF5 mouse models

Mouse models have been the most definitive way to clarify the physiological role of a gene in development and diseases. Several Klf5 knockout and transgenic mouse models have been established for studying KLF5 function.

Klf5 knockout mice

Based on the studies of Klf5 knockout mouse models, Klf5 has been implicated in pathological and physiological processes including embryonic development, angiogenesis, adipose tissue development, cartilage degradation during skeletal development, energy metabolism, and lung morphogenesis.

Conventional Klf5 homozygous knockout mice died before embryonic day 8.5, indicating that Klf5 is essential for mouse embryonic development. The Klf5 heterozygous knockout mice show diminished levels of arterial-wall thickening, angiogenesis, cardiac hypertrophy, and interstitial fibrosis in response to external stress, suggesting a role of KLF5 in linking external stress and cardiovascular remodeling [59]. The heterozygous KLF5 knockout mice exhibit a marked deficiency in white adipose tissue development [46] and skeletal growth retardation in the perinatal period [100]. While infection of WT mice with bacteria increases the heights of colonic crypt and the expression of KLF5, the KLF5 (\pm) mice show an attenuated induction of hyperproliferative responses after bacteria infection [101].

Recently, Wan et al. reported a conditional Klf5 knockout mouse model [41]. In this study, Klf5 was found to be essential for lung morphogenesis and function [41]. When Klf5 is specifically knocked out in respiratory epithelial cells in the fetal lung, lung maturation is inhibited during the saccular stage of development, and phenotypic abnormalities occur in different components including the respiratory epithelium, the bronchiolar smooth muscle, and the pulmonary vasculature. Mice with both Klf5 alleles knocked out die of respiratory distress immediately after birth [41]. A set of genes are abnormally regulated by the knockout of KLF5 [41].

Klf5 transgenic (Tg) mice

Besides knockout mouse models, two tissue-specific Klf5 Tg mouse models have been developed [85, 102]. Klf5 has been found to regulate esophageal basal epithelial cell proliferation and stemness of epidermis.

Klf5 has been shown to promote basal epithelial cell proliferation but is not sufficient to produce tumors [102]. Using the ED-L2 promoter of the Epstein–Barr virus, Goldstein et al. expressed KLF5 throughout esophageal epithelia in mice, and examined the role of KLF5 in the proliferation of esophageal cells. While there was no evidence of esophageal dysplasia or cancer, staining for bromodeoxyuridine (BrdU) demonstrated increased proliferation in basal cells but not in suprabasal cells, and did not appear to affect the differentiation of esophageal epithelium [102].

KLF5 has a role in the homeostasis of epidermis and skin morphogenesis. Expression of KLF5 is at relatively higher levels in keratinocytes throughout the adult human epidermis. Expression is stronger in the matrix and the inner root sheath cuticle layer of the hair follicle, sebaceous glands, and sweat glands [44]. Using the keratin 5 promoter, Sur et al. [85] specifically expressed KLF5 in the basal layer of the epidermis, and demonstrated that KLF5 affects epidermal development and disrupts epithelial–mesenchymal interactions necessary for skin adnexae formation as well as craniofacial morphogenesis during embryogenesis. The transgenic mice exhibit exencephaly, craniofacial defects, persistent abdominal herniation, and ectodermal dysplasia. In addition, the epidermis is hypoplastic and undergoes abnormal differentiation with expression of keratin 8, a marker for single-layered epithelia, in the stratified epidermis. Overexpression of KLF5 in adult mice leads to hyperkeratosis, follicle occlusion, and epidermal erosions. Furthermore, a decrease in stem cell population of bulge keratinocytes has been noticed, as characterized by the expression pattern of integrin $\alpha 6$ and CD34 markers.

Taken together, these in vivo studies using the knockout and transgenic techniques support an important role of KLF5 in embryonic and tissue development through regulating cell proliferation, differentiation, and stemness.

KLF5 and diseases

Cancer

Expression of KLF5 was found to be abnormal in many cancer types. Functional studies also support that KLF5 is an important cancer-related gene (Table 3). Based on its positive role in cell proliferation and survival, KLF5 has been suggested to be oncogene. However, some genetic, expression, and functional studies imply that KLF5 could be a tumor suppressor under some scenarios. It is attempting to speculate that KLF5 has context-dependent functions in carcinogenesis.

The *KLF5* gene undergoes frequent genetic alteration in different cancer types including prostate, breast, and salivary gland tumors. The *KLF5* gene is rarely mutated in a large number of prostate and breast cancer cell lines [78, 84]. The *KLF5* gene locus (13q21) is the second most frequent deletion in different types of human cancers according to a large number of comparative genomic hybridization (CGH) studies [103]. The deletion of 13q21 associates with metastases and higher tumor grade in prostate cancer [78, 104–107]. Analysis of human tumors demonstrated that *KLF5* centers the deletion at 13q21 in prostate cancer. The majority of *KLF5* deletions in prostate

Table 3 The role of KLF5 in different types of cancer

Cancers	Genetic and expression	Function	References
Intestine and colon	mRNA expression is decreased in APC ^{min} mouse adenomas and familial adenomatous polyposis	Promotes cell proliferation	[51, 73, 74, 79, 101]
Breast	Gene copy number loss. mRNA is low in ER + cancer cell lines, high in ER-tumors	Promotes MCF7 xenograft growth	[36, 84, 110]
Prostate	Gene copy number loss. mRNA is lost in some cancer cell lines	Inhibits DU145 and 22Rv1 colony formation	[78, 111]
Bladder	Overexpression in some prostate cancers mRNA is upregulated and downregulated in cancer cell lines but not in tumor samples	Promotes TSU-Pr1 xenograft growth	[20, 40]
Leukemia	mRNA is upregulated and downregulated in cancer cell lines and acute lymphoblastic leukemia by Northern blot	Promotes EU-4 resistance to doxorubicin	[45]
Esophageal	mRNA level is high in stem-like cancer cells by qRT-PCR	Inhibits cell proliferation, survival, and invasion. Promotes basal cell proliferation in KLF5 Tg mice	[77, 102, 112]
Salivary gland	Gene copy number gain by CGH		[108]
Gastric cancer	Protein expression is high in early-staged, lymph node metastasis negative, and small sized tumors by IHC		[109]
Nasopharyngeal	mRNA is down-regulated by microarray		[138].
Melanoma	mRNA is down-regulated in two Ras mutated cancer cell lines by microarray		[139]

cancer are hemizygous deletion equivalent to haploinsufficiency [59], which inactivates *KLF5* by reducing *KLF5* expression. Consistently, *KLF5* is excessively degraded in human cancer cell lines by overexpressed WWP1 E3 ligase [31–33]. However, a chromosomal region at 13q spanning the *KLF5* locus appears to be amplified in some salivary gland tumors, as detected by CGH [108]. Thus, while deletion at *KLF5* is frequent, it can also be amplified in human cancer.

Consistent with the genetic alterations, frequent expression aberrations for *KLF5* have been well documented. Chen et al. [78, 84] found that the expression of *KLF5* mRNA is frequently reduced or absent in many cell lines from both breast cancer and prostate cancer compared to immortalized cell lines. Similarly, in intestinal tumors and adenomatous polyposis adenomas, reduced expression of *KLF5* mRNA occurs [79]. Even in Ras-mediated transformation of IEC-18 and IMCE cells, *KLF5* is markedly downregulated [79]. In another study, analysis of 247 gastric carcinomas by immunohistochemical staining showed that *KLF5* is expressed in 46% (113/247) of tumor tissues, and expression of *KLF5* is more frequently detected in early stage tumors than in late stage tumors (63 vs. 38%), in tumors without lymph node metastasis (54 vs. 40%), and in tumors smaller than 5 cm in size (53 vs. 38%)

[109]. In contrast, Tong et al. [110] examined the expression of *KLF5* mRNA by qPCR in breast cancer, and found a significant correlation between increased *KLF5* expression and reduced disease-free and overall survival in patients with breast cancer. *KLF5* expression also appears to positively correlate with HER-2 and MKI67 markers of breast cancer and negatively correlate with patient age at diagnosis [110]. In human prostate cancer tissues, Chaib et al. [111] reported that *KLF5* mRNA is upregulated in prostate cancers compared to normal tissues. Stem-like esophageal cancer cells, as defined by the side population, have a higher level of *KLF5* expression than other cells [112]. More independent studies in primary tumors with different approaches are required to clarify these inconsistent results. Nevertheless, the expression of *KLF5* in tumors appears to be altered during tumor development.

Functional studies in vitro have shown inconsistent results in different cell lines. For example, in the TSU-Pr1 bladder cancer cell line, expression of *KLF5* promotes cell proliferation [40]. Consistently, *KLF5* promotes survival and drug resistance in the HCT116 colon cancer cell line [80]. Furthermore, knockdown of *KLF5* could inhibit the multicellular tumor spheroid formation in vitro [113]. In contrast, *KLF5* has shown a growth inhibitory function in vitro in some cancer cell lines tested, including those from

the colon [79] and prostate [78]. In the TE2 esophageal cancer cell line, which was derived from a poorly differentiated esophageal squamous carcinoma, stable expression of KLF5 inhibits cell proliferation and invasion, and decreases cell survival [77].

Several *in vivo* studies suggest that KLF5 promotes tumorigenesis. Chen et al. [40] directly demonstrated that expression of KLF5 promotes TSU-Pr1 bladder cancer cell line tumorigenesis in SCID mice. Similarly, expression of KLF5 in the MCF7 breast cancer cell line also promotes xenograft growth in nude mice in the presence of estrogen (unpublished results). Shindo et al. showed that the angiogenesis of transplanted sarcoma 180 is attenuated in the *Klf5* (\pm) mice [59]. Similarly, the crypt cell proliferation in the colon in response to pathogenic bacterial infection is also reduced in the *Klf5* (\pm) animals [101]. Consistently, *Klf5* promotes esophageal basal epithelial cell proliferation although it is not sufficient to produce tumors [102]. Based on these *in vivo* research results, KLF5 could have context-dependent functions in oncogenesis.

Based on the recent discovery of KLF5 as an essential cofactor for TGF β in epithelial cells [30], it is likely that the role of KLF5 in tumorigenesis is similar to that of TGF β signaling, being a tumor suppressor in early stage but a tumor promoter in late stage tumorigenesis [114–116]. We are currently addressing this outstanding question using knockout mouse models.

Cardiovascular diseases

KLF5 has been best studied in the physiology and pathology of VSMCs. Based on a large number of expression and functional analyses, KLF5 contributes to all VSMC-related diseases, such as atherosclerosis, restenosis after angioplasty, cardiac hypertrophy, and hypertension, by regulating the expression of genes including SMemb/NMHC-B, SM22 α , Egr-1, PDGF, and others (Table 1).

KLF5 is preferentially expressed in proliferating SMCs but reduced in differentiated cells under physiological and pathological conditions. It has been reported that KLF5 is abundantly expressed in embryonic smooth muscles and is downregulated with vascular development [117, 118]. Consistently, KLF5 is abundantly expressed in fetal but not in adult aortic SMCs of humans and rabbits [119]. The expression of KLF5 is increased in the neointimal smooth muscles in response to vascular injury [118]. In atherectomy specimens from primary and restenotic lesions, predominant expression of KLF5 was detected in SMCs. In addition, restenotic lesions expressing higher levels of KLF5 show higher incidence of restenosis than lesions without [119]. In cultured SMCs from atherectomy specimens obtained from patients with coronary restenosis after

angioplasty, Sakamoto et al. [120] also found a correlation between KLF5 expression and SMC activity in outgrowth.

KLF5 also plays a role in the development of cardiac allograft vascular disease. In rats, Ogata et al. performed heterotopic cardiac transplantation, and examined KLF5 expression by IHC in all grafts. From 4 to 8 weeks after transplantation, SMCs showed positive staining for KLF5 in diffusely thickened coronary arteries and the perivascular space, and the level of KLF5 expression was significantly higher in allografts compared to isografts [121]. Using a heterotopic abdominal heart transplant model in monkeys, Wada et al. [122] showed that KLF5 and Egr-1 are induced in VSMCs of rejected cardiac allografts well before morphologic changes, such as intimal thickening, can be detected, suggesting that expression of KLF5 is one of the initial events in allograft angiopathy.

Additional studies provide direct evidence for the role of KLF5 in the modulation of cardiac hypertrophy and hypertension. Angiotensin II plays a critical role in cardiovascular remodeling through KLF5, as knockout of KLF5 in mice attenuates angiotensin II-induced cardiac hypertrophy and fibrosis [59]. Knockout of KLF5 also diminishes levels of smooth muscle and adventitial cell activation [59]. In a spontaneous hypertensive rat model, VSMCs change to the synthetic phenotype, which involves the complement 3 (C3) gene. Yao et al. [123] found that C3 increases the transcription of KLF5 in SMCs.

Other diseases

KLF5 may play a role in inflammatory diseases. The pro-inflammatory factors TNF α and LPS induce KLF5 expression in human umbilical vein endothelial cells, venous SMCs, and intestinal epithelial cells [60, 124]. The expression induction of monocyte chemoattractant protein-1 (MCP-1) by TNF α depends on KLF5, as knockdown of KLF5 by RNAi inhibits the effect of TNF α on MCP-1 expression [124]. Similarly, knockdown of KLF5 by RNAi reduced the expression of p50 and p65 subunits of NF- κ B and its downstream target genes, TNF α and IL-6, in response to LPS [56]. Finally, KLF5 has been shown to directly interact with NF- κ B in epidermal epithelial cells [44].

KLF5 may also have a role in obesity. Recently, a role of KLF5 in energy metabolism has been established. Oishi et al. [38] found that mice with one *Klf5* allele knocked out are resistant to high fat-induced obesity despite consuming more food than WT mice. This effect appears to be mediated by the function of KLF5 in gene regulation, as expression of genes involved in lipid oxidation and energy uncoupling is upregulated in *Klf5* (\pm) mice.

Involvement of KLF5 in additional pathological processes has also been reported. During intestinal obstruction,

which causes dramatic phenotypic changes in intestine smooth muscles, expression of KLF5 initially increases but then decreases [125]. KLF5 is among the genes downregulated in human tissues from ulcerative colitis, an inflammatory bowel disease [126]. In addition, KLF5 could be involved in the pathophysiology of schizophrenia [23].

Conclusions

In summary, KLF5 is an essential transcription factor that regulates the transcription of a large number of genes in different contexts. The transcription of KLF5 itself is also regulated by different signaling pathways, including MAPK, PKC, and PI3 K, generally with an increased expression during cell proliferation and a decreased expression during differentiation, although it is also necessary for signaling-induced differentiation. Different protein modifications including phosphorylation, acetylation, ubiquitination, and sumoylation, which could be mediated by different signaling pathways, occur on KLF5 protein and regulate its transactivation activities and expression levels. Biologically, KLF5 appears to be essential not only for the proliferation of different types of cells including stem and progenitor cells but also for the differentiation of progenitor cells, which likely underlies the function of KLF5 in such physiologic and pathologic processes as epithelial homeostasis, development, tumorigenesis, cardiovascular remodeling, inflammation, and apoptosis. The expression of KLF5 is frequently altered in human diseases, including cancer and cardiovascular diseases.

Perspectives

Although KLF5 is a key transcription factor involved in multiple signaling cascades, whether the KLF5 pathway can be developed as a diagnostic tool and a therapeutic target remains to be elucidated. It is important to further understand KLF5 regulation and mechanisms of action under physiological and pathological conditions, to develop reagents and methods to accurately and conveniently measure KLF5 expression and activity changes in pathological specimens, and to develop treatments targeting the KLF5 pathway.

Although our biochemical and cellular knowledge of KLF5 has rapidly increased in the last decade as reviewed above, the roles of KLF5 in normal tissue development and disease progress are still far from clear. The ubiquitous expression of KLF5 in different types of cells and tissues, the diversified posttranslational modifications and interacting proteins, the numerous context-dependent target genes, and redundancy among the KLF5 family members contribute to the complexity of KLF5 study. It is important

to develop inducible and tissue-specific animal models of KLF5 expression for human diseases because KLF5 is temporally and spatially regulated under physiological and pathological conditions.

KLF5 has potential as a biomarker for diagnosis and prognosis in cancers and cardiovascular diseases. The expression of KLF5 mRNA associates with reduced survival in patients with breast cancer [110]. KLF5 expression also appears to correlate positively with a higher incidence of restenosis [119]. Given that IHC is widely used in the clinic for diagnosis and prognosis, it is necessary to establish the standard IHC techniques for KLF5 to evaluate the potential of KLF5 as useful clinical biomarker or as a potential stand-alone prognostic factor. So far, KLF5 protein expression has only been analyzed by IHC in gastric cancer [109].

Although multiple anti-KLF5 antibodies are commercially available, the specificity appears to be unsatisfactory. Future studies focusing on accurately measuring KLF5 expression and activity changes in a large number of pathological specimens will be necessary.

Transcription factors are usually not ideal for targeted therapy using small molecular inhibitors. However, KLF5 directly interacts with RAR α to regulate downstream target genes in cardiovascular remodeling [127]. The synthetic RAR α agonist Am80 was shown to inhibit the activity of KLF5 [43, 118]. Acyclic retinoid (ACR) also attenuates the functional interaction of KLF5 and RAR α [127]. These small molecules have the potential to be developed as drugs for cardiovascular diseases. With the development of siRNA delivery technology, anti-KLF5 siRNAs could also be developed into drugs. Additionally, several RTK and PI3 K small molecular inhibitors (AG17, LY294002, and Wortmannin) were identified as inhibiting KLF5 transcription and colon cancer cell proliferation by cell-based high throughput screening [73]. Once the KLF5 pathway is clearly understood, the positive upstream regulators (e.g., protein kinases) and downstream target proteins (e.g., FGF-BP) could be inhibited by small molecular inhibitors or monoclonal antibodies.

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PMEPA1 promotes androgen receptor-negative prostate cell proliferation through suppressing the Smad3/4–c-Myc–p21^{Cip1} signaling pathway

Rong Liu,¹ Zhongmei Zhou,^{1,2} Jian Huang³ and Ceshi Chen^{1,2*}

¹ The Center for Cell Biology and Cancer Research, MS355, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208, USA

² Key Laboratory of Animal Models and Human Disease Mechanisms of Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Kunming, Yunnan, China, 650223

³ Lester and Sue Smith Breast Center, Baylor College of Medicine, BCM MS 600, One Baylor Plaza, Houston, TX 77030, USA

*Correspondence to: Ceshi Chen, The Center for Cell Biology and Cancer Research, MS355, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208, USA. e-mail: chenceshi@hotmail.com

Abstract

The *PMEPA1* gene has been shown to suppress the androgen receptor (AR) and TGF β signaling pathways and is abnormally expressed in prostate tumours. However, the role and mechanism action of *PMEPA1* in AR-negative prostate cancer are unclear. Here, we demonstrate that inhibition of *PMEPA1* suppresses AR-negative RWPE1 and PC-3 prostate cell proliferation through up-regulating the p21 transcription. Additionally, *PMEPA1* overexpression suppresses the p21 expression and promotes cell proliferation. *PMEPA1* is induced by TGF β as a negative feedback loop to suppress Smad3 phosphorylation and nuclear translocation; up-regulates c-Myc; down-regulates p21; and promotes PC-3 cell proliferation. The *PMEPA1* functions depend on its Smad2/3 binding motif. Consistently, depletion of Smad3/4, but not Smad2, blocks *PMEPA1*'s functions of regulating c-Myc and p21. Importantly, stable depletion of *PMEPA1* in PC-3 inhibits xenograft growth. Finally, we found that *PMEPA1* is overexpressed in a subset of prostate cancer cell lines and tumours. These findings suggest that *PMEPA1* may promote AR-negative prostate cancer cell proliferation through p21.

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Introduction

The *PMEPA1* (*TMEPA1*, *STAG1*, *ERG1.2* or *N4WBP4*), prostate transmembrane protein, androgen induced 1, gene is located at chromosome 20q13, a region frequently amplified in multiple solid tumours, including prostate [1], breast [2], and colon [3] cancers. *PMEPA1* has been shown to be an androgen-induced gene predominantly expressed in the prostate and over-expressed in androgen-independent xenografts [4,5]. Consistently, *PMEPA1* mRNA expression is increased in a more malignant prostate cancer PC-3 subclone [6]. In agreement with the 20q13 amplification, *PMEPA1* is overexpressed in breast and ovarian cancers [5], colon cancer [7–9], renal cell carcinoma [9], and stomach adenocarcinoma [9]. Besides androgen, many growth factors, such as transforming growth factor β (TGF β) [7], EGF, IGF, PDGF [5], and GM-CSF [10], can induce *PMEPA1* expression.

TGF β is well known to play important roles in embryo and cancer development by controlling cell growth, differentiation, and migration. TGF β initiates

signaling by assembling a receptor serine/threonine kinase complex that phosphorylates Smad2 and Smad3 [11]. Following that, Smad2/3 are translocated into the nucleus and form transcription complexes with Smad4 to regulate the transcription of target genes, such as *c-Myc* [12] and the cyclin-dependent kinase inhibitors *p21^{Cip1}* (p21), *p27^{kip1}* (p27) [13], and *p15^{Ink4b}*. Thus, TGF β inhibits normal epithelial cell proliferation and induces differentiation. Recently, *PMEPA1* mRNA has been shown to be induced by TGF β [7,14–16]. Interestingly, *PMEPA1* has been suggested to suppress TGF β signaling through binding to Smad2/3 and preventing them from phosphorylation by the TGF β receptor kinase complex [15].

However, *PMEPA1* has also been shown to have tumour suppressor activities. For example, *PMEPA1* was reported to mediate p53-dependent apoptosis [17] and to inhibit prostate cancer cell colony formation *in vitro* [18,19]. Xu *et al* reported that the *PMEPA1* mRNA levels are down-regulated in a subset of prostate tumours [18]. Recently, *PMEPA1* has been shown to suppress androgen receptor (AR)-positive LNCaP prostate cell proliferation through recruiting

the NEDD4 ubiquitin E3 ligase to target AR for degradation [19]. Thus, the physiological function and mechanistic action of PMEPA1 in prostate cancer are controversial.

In this study, we show that PMEPA1 promotes AR-negative prostate cancer cell proliferation and tumorigenesis through suppressing the expression of p21. PMEPA1 directly decreases Smad3 nuclear translocation in the absence and presence of TGF β in PC-3. Depletion of Smad3 or Smad4 abrogates PMEPA1's regulation on *c-Myc* and *p21* expression. Myc partially contributes to PMEPA1-induced *p21* transcription suppression. Finally, the PMEPA1 β protein is overexpressed in a subset of prostate cancers. These new findings suggest that PMEPA1 promotes AR-negative prostate cell proliferation through suppressing the Smad3/4–c-Myc–p21 signaling pathway.

Materials and methods

Cell lines

The AR-negative immortalized RWPE1 prostate epithelial cell line [20] and the PC-3 prostate cancer cell line [21] were purchased from American Type Culture Collection (ATCC). RWPE1 was maintained in DMEM/Ham's F-12 50/50 medium supplemented with 5% horse serum, 0.5 μ g/ml hydrocortisone, 10 μ g/ml insulin, 20 ng/ml epidermal growth factor (EGF), 0.1 μ g/ml cholera enterotoxin, 1% penicillin and streptomycin (PS), and 2 mm L-glutamine. PC-3 was cultured in Ham's 12-K medium containing 5% fetal bovine serum (FBS), 1.5 g/l sodium bicarbonate, and 1% PS. HEK293FT cells were cultured in DMEM containing 5% FBS and 1% PS. All cells were maintained in a humidified atmosphere with 5% CO₂ at 37 °C.

Transfection

All siRNAs and plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The siRNA target sequences are provided in the Supporting information, Supplementary Table 1. The pre-designed siRNAs were purchased from Ambion, Inc (Austin, TX, USA). The luciferase control siRNA

was synthesized by Thermo Fisher Scientific Inc (Dharmacon, Lafayette, CO, USA). The final siRNA concentration was 20 nm.

Antibodies

The anti-PMEPA1 antibody was purchased from Novus Biologicals, Inc (Littleton, CO, USA); the anti-c-Myc antibody was from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA); and the anti-p27 antibody was from BD Bioscience (San Jose, CA, USA). The anti-p21, anti-Smad3, anti-pSmad3^{S423/425}, anti-Smad2, anti-Samd4, anti- α / β -tubulin, anti-PARP antibodies, and all secondary antibodies were from Cell Signaling (Danvers, MA, USA).

PMEPA1 immunohistochemistry (IHC) staining

The anti-PMEPA1 antibody (Novus; 1:150 dilution) was used for IHC after validation and optimization using PMEPA1 knockdown PC3 cells. A high-density (114 cases/208 cores) prostate adenocarcinoma (grade 1–4) and normal prostate tissue array were purchased from US Biomax (Rockville, MD, USA). The pathology diagnosis and grade information for 93 primary prostate tumours are available online (<http://www.biomax.us/tissue-arrays/Prostate/PR2085a>). Antigens were retrieved by boiling the slide in 0.1 m Tris–HCl, pH 9.0 buffer for 5 min. A standard DAB staining protocol was used. Immunostained slides were scored using the 'Allred score' method [22]. This study was approved by IRB at Albany Medical College.

Lentiviral shRNA and cDNA transduction

A lentiviral pSIH-H1-Puro vector was used to express shRNAs by following the manufacturer's manual. The PMEPA1 shRNAs were designed according to the PMEPA1 siRNA target sequences. The luciferase shRNA was used as a negative control. The human *PMEPA1* gene was amplified with the pfu enzymes by PCR. The PCR products were digested by *Bam*H I/*Xho* I, subcloned into the pLenti6/V5-D-TOPO vector (Invitrogen), and verified by DNA sequencing. The PPNR residues were replaced with AAAA in PMEPA1 β -PPNRm by using a PCR-mediated mutagenesis method. The PCR primer sequences used in this

Table 1. The PMEPA1 IHC results

Allred scores*	Normal	Adjacent	Grade 1	Grade 2	Grade 3	Grade 4
Negative (0–2)	7	5	7	12	9	14
Weak (3–4)	2	4	4	2	11	5
Medium (5–6)	1	1	4	8	6	3
Strong (7–8)	0	0	1	0	6	1
All positive	3 (30%)	5 (50%)	9 (56%)	10 (45%)	23 (72%)†	9 (39%)
Total	10	10	16	22	32	23

*The 'Allred score' method [22]: a proportion score was assigned representing the estimated proportion of positive staining tumour cells (0 = none; 1 < 1/100; 2 = 1/100 to 1/10; 3 = 1/10 to 1/3; 4 = 1/3–2/3; 5 = >2/3). The average estimated intensity of staining in the positive cells was assigned an intensity score (0 = none; 1 = weak; 2 = intermediate; 3 = strong). The proportion score and intensity score were added to obtain a total score ranging from 0 to 8. Based on the total score, the case was defined as negative (0–3), weak positive (4–5), intermediate positive (6), or strong positive (7–8). Tumours that were positive for PMEPA1 were defined as having a score greater than 3. † $p < 0.05$, Fisher's exact test compared with normal and adjacent prostate tissues.

study are listed in the Supporting information, Supplementary Table 2. The pLenti6/V5-GW/*lacZ* vector was used as a negative control.

All lentiviral plasmids and the packing plasmids were co-transfected into HEK293FT cells using Lipofectamine 2000. Lentiviruses were collected at 72 h after transfection and used to transduce PC-3 cells in a 24-well plate. Twenty-four hours after transduction, blasticidin (15 µg/ml) (for the pLenti6 system) and puromycin (2 µg/ml) (for the pSIH-H1-Puro shRNA system) were added to select drug-resistant cell populations.

Tumourigenesis in nude mice

Twelve 5- to 6-week-old male SCID hairless outbred (SHO) mice (Charles River, Wilmington, MA, USA) were used for this study (six mice for each group). Two and a half million PC-3 (Lucsh and PMEPA1sh#2) cells per site (two sites for each mouse) were subcutaneously injected into the SHO mice. The tumour volume was measured once a week from day 14. The tumour volume was calculated as $\pi/6 \times \text{length} \times \text{width}^2$. All mice were sacrificed at day 70 and the final tumour weights were recorded.

Results

PMEPA1 depletion inhibits cell proliferation and blocks the G1/S cell cycle transition

Two different siRNAs targeting different regions of *PMEPA1* mRNA were used to knock down PMEPA1 in two AR-negative prostate cell lines including RWPE1 and PC-3 (Figure 1A). DNA synthesis was dramatically suppressed in both cell lines. To test whether knockdown of PMEPA1 alters the cell cycle, we examined the PC-3 cell cycle by flow cytometry. As shown in Figure 1B, knockdown of PMEPA1 significantly decreased cells in the S phase and increased cells in the G1 phase simultaneously. Thus, it appears that depletion of endogenous PMEPA1 inhibits cell proliferation through blocking the G1/S cell cycle transition.

Depletion of endogenous PMEPA1 inhibits cell proliferation through up-regulating p21 expression

Since knockdown of PMEPA1 blocks the G1/S cell cycle transition, the expression levels of several cell cycle proteins, including cyclin D1, cyclin B1, p21, p27, and p15^{Ink4b}, were examined by immunoblotting in RWPE1 and PC-3. The p21 protein expression levels were up-regulated by both PMEPA1 siRNAs in both cell lines (Figure 2A). The p27 expression levels were only significantly up-regulated in RWPE1 but not in PC-3 (Figure 2A). Other proteins did not show significant expression changes (data not shown). In addition, PMEPA1 depletion also up-regulated the p21 protein levels in HeLa (Supporting information, Supplementary Figure 1A). However, PMEPA1 knockdown did

not up-regulate the p21 protein levels in AR-positive 22Rv1 and LNCaP prostate cancer cell lines (data not shown).

To characterize the primary mechanism by which PMEPA1 regulates p21 protein expression in prostate cells, we examined *p21* mRNA expression by qRT-PCR and found that both PMEPA1 siRNAs up-regulated the *p21* mRNA levels in RWPE1 and PC-3 (Figure 2B). PMEPA1 did not affect degradation of *p21* mRNA and protein (Supporting information, Supplementary Figures 1B and 1C). These results indicate that PMEPA1 may regulate *p21* gene transcription.

p21 and p27 are well-known tumour suppressors that inhibit G1/S cell cycle progression and cell proliferation. To test whether PMEPA1 depletion suppresses cell proliferation through up-regulating p21 and p27 in prostate cells, we depleted p21 and p27 respectively in both RWPE1 and PC-3 (Figure 2C) and examined whether PMEPA1 knockdown can still inhibit DNA synthesis. Importantly, depletion of p21, but not p27, completely rescued the PMEPA1 knockdown-induced decrease in DNA synthesis (Figure 2D). The same results were obtained from another p21 siRNA in both cell lines (Supporting information, Supplementary Figure 2). These results suggest that knockdown of PMEPA1 inhibits cell proliferation through up-regulating p21 in AR-negative prostate cells (Supporting Information, Supplementary Materials and Methods).

PMEPA1 overexpression down-regulates p21 and promotes cell proliferation

To further confirm that PMEPA1 regulates *p21* gene expression in prostate cells and to characterize the possible mechanism by which PMEPA1 regulates p21 protein expression, we overexpressed wild-type (WT) PMEPA1 (β and α , Figure 3A) in PC-3. Multiple bands for exogenous PMEPA1 were detected possibly because of glycosylation [5] and alternative translation initiation sites [9]. As expected, PMEPA1 decreased p21 protein expression levels, compared with the LacZ control in PC-3 (Figure 3B). PMEPA1 β is more potent than PMEPA1 α , which has a shorter N-terminal fragment before the transmembrane (TM) domain (Figure 3A). Moreover, PMEPA1 β overexpression can rescue the PMEPA1 siRNA-induced p21 protein expression in PC-3 (Supporting information, Supplementary Figure 3A), suggesting that p21 up-regulation by the PMEPA1 siRNA is specific.

In a previous study, the PMEPA1 α isoform localized in punctuate clusters concentrated around the nucleus, whereas the PMEPA1 β isoform was distributed in a diffuse pattern in the cytoplasm [7]. Watanabe *et al* further demonstrated that PMEPA1 was localized in the Golgi apparatus and endosomes in MCF10A1 cells by electron microscopy [15]. To understand why PMEPA1 β is more potent than PMEPA1 α for p21 expression suppression, we examined their subcellular localizations in PC-3 by direct immunofluorescence.

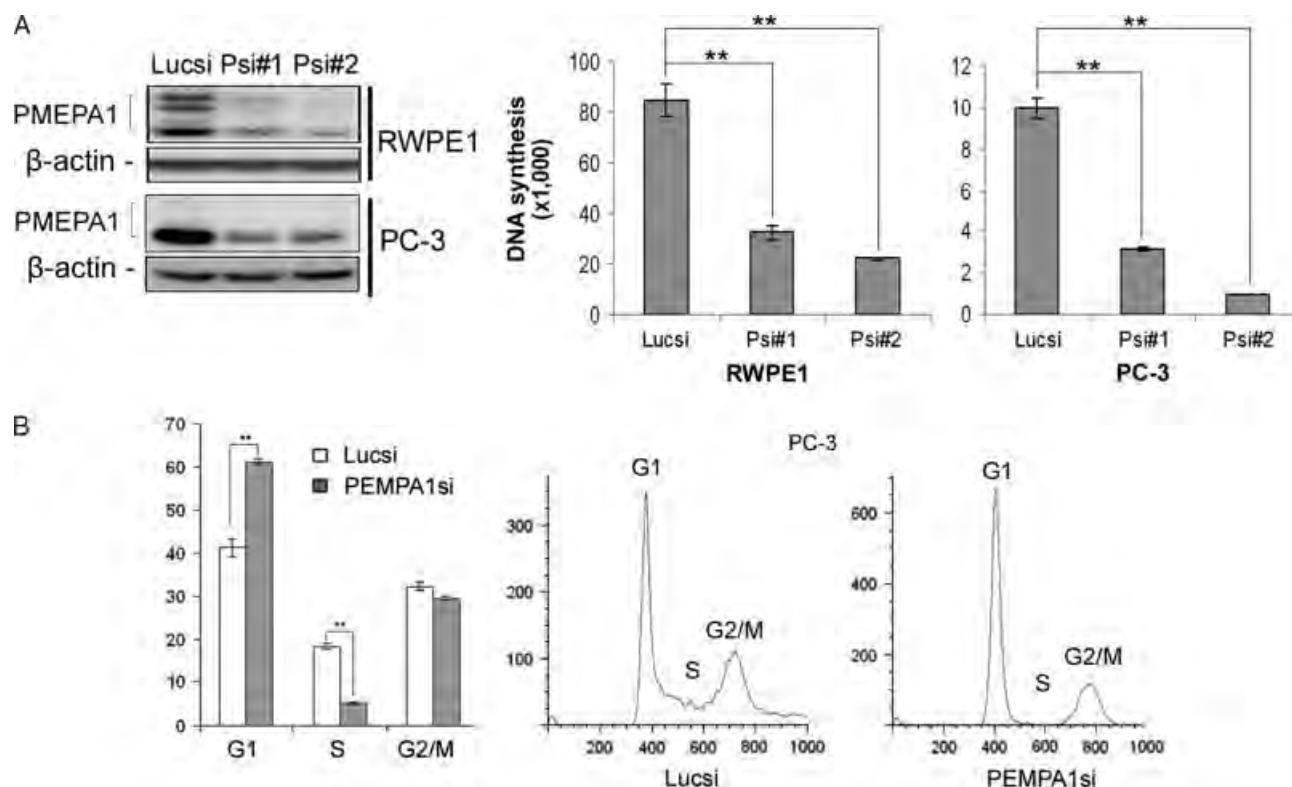


Figure 1. Depletion of PMEPA1 inhibits AR-negative prostate cell proliferation through blocking the G1/S cell cycle transition. (A) Depletion of PMEPA1 inhibits [3 H]thymidine incorporation in both RWPE1 and PC-3 prostate cell lines. Knockdown of PMEPA1 protein expression by two different siRNAs in RWPE1 and PC-3 was examined by immunoblotting (left panel). (B) Knockdown of PMEPA1 by siRNA#2 in PC-3 prevents G1/S cell cycle progression by the PI staining and flow cytometry analysis. The average results from three experiments are shown on the left-hand side. ** $p \leq 0.01$ (t -test). One example result of flow cytometry is shown on the right-hand side.

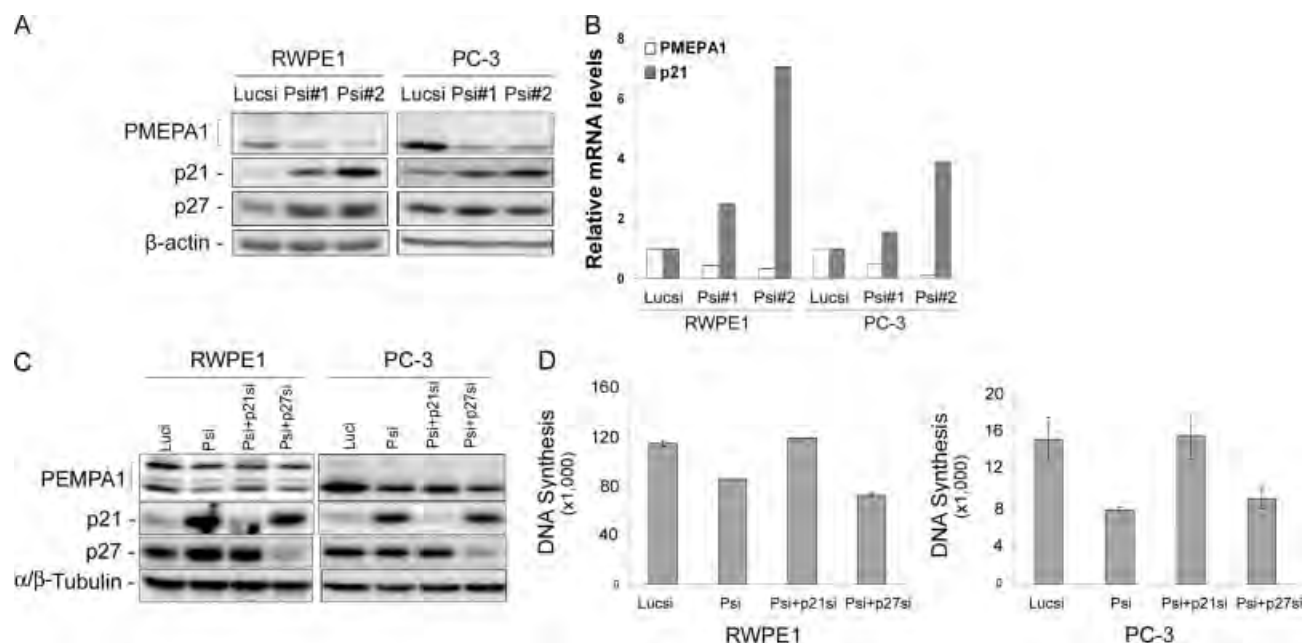


Figure 2. Endogenous PMEPA1 promotes prostate cell proliferation through suppressing p21 expression. (A) Knockdown of PMEPA1 by two different siRNAs increases the p21 protein expression levels in RWPE1 and PC-3, as examined by immunoblotting. p27 is also up-regulated in RWPE1 but not in PC-3. (B) Knockdown of PMEPA1 by two different siRNAs increases the p21 mRNA expression levels in both RWPE1 and PC-3, as determined by qRT-PCR. GAPDH was used as the loading control. The *PMEPA1* mRNA levels are also decreased by two different siRNAs. (C) Knockdown of p21 and p27 in RWPE1 and PC-3 was examined by immunoblotting. (D) Knockdown of p21, but not p27, can completely rescue the PMEPA1siRNA#1 causing cell growth arrest in both RWPE1 and PC-3. Data are presented as the mean value and standard deviation from three independent experiments.

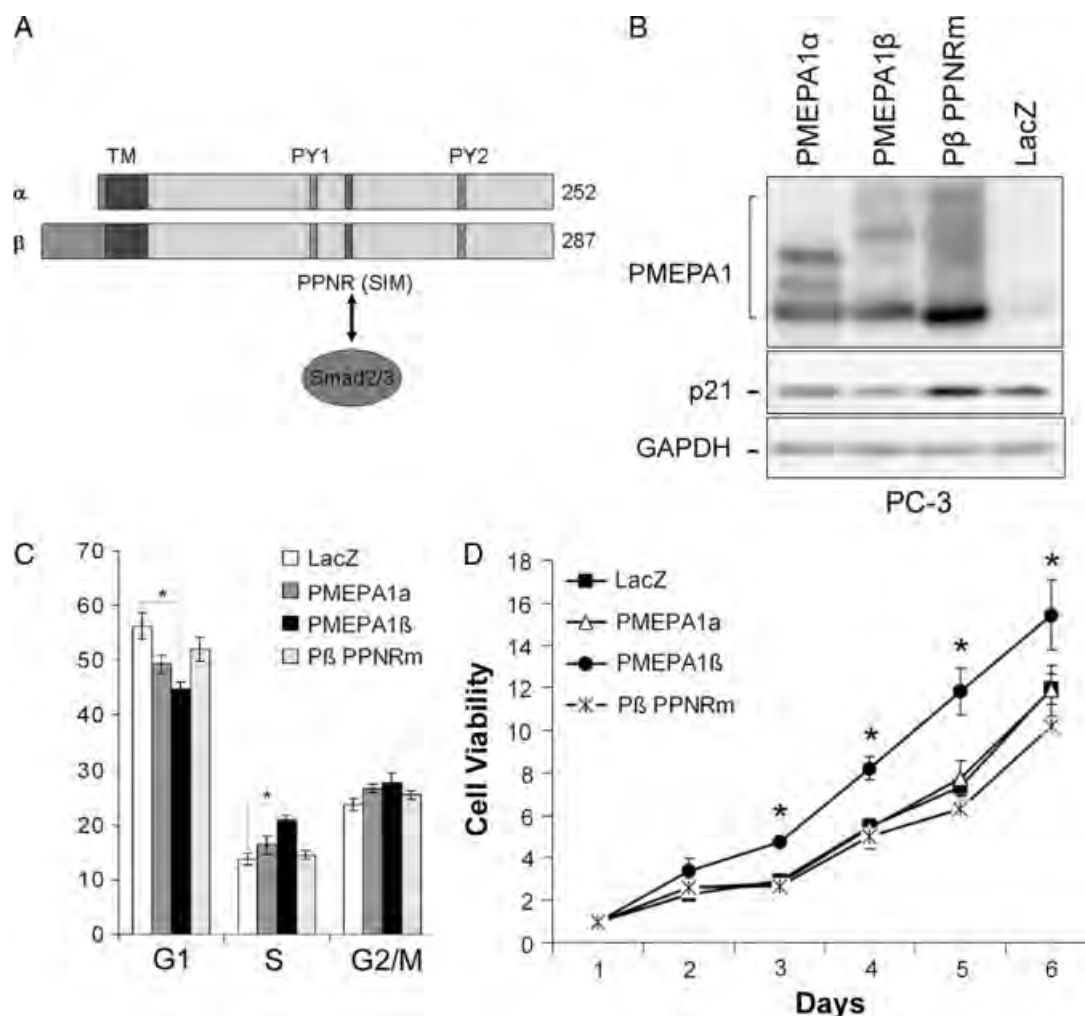


Figure 3. Forced overexpression of PMEPA1 promotes PC-3 cell proliferation and inhibits p21 expression in a SIM-dependent manner. (A) Diagrams of two PMEPA1 protein isoforms that differ in their N-terminuses. TM = transmembrane domain. Two PY motifs and one SIM (Smad interacting motif) are shown. (B) Stable overexpression of the WT PMEPA1β isoform, but not PPNR-mutated PMEPA1β, by lentiviruses significantly decreases the p21 protein level in PC-3. The PMEPA1α isoform is much less efficient in suppressing p21 protein expression compared with the PMEPA1β isoform. GAPDH was used as the loading control. (C) Overexpression of the PMEPA1β isoform promotes PC-3 G1/S cell cycle progression. PC-3 cell populations stably expressing LacZ, PMEPA1α, β, and PMEPA1β-PPNRm were analysed. (D) Stable overexpression of PMEPA1β promotes PC-3 cell growth by the SRB assay in the course of 6 days. * $p \leq 0.05$ (t-test).

The GFP fluorescence protein was fused to the C-terminus of PMEPA1 isoforms, and they were transfected into PC-3. Indeed, the subcellular localizations of the two isoforms were slightly different. The β isoform was distributed more diffusely than α (Supporting information, Supplementary Figure 3B). This might explain the functional differences of these two variants.

Both PMEPA1 isoforms contain the Smad interacting motif (SIM, PPNR) that can bind to Smad2/3 to block TGFβ signaling [15] (Figure 3A). It is well known that TGFβ can suppress cell proliferation through inducing *p21* gene transcription [23]. To test whether PMEPA1 regulates *p21* through Smad2/3, we mutated PPNR into 4A in PMEPA1β. We first confirmed that PMEPA1β-PPNRm cannot interact efficiently with Smad2/3 by the co-immunoprecipitation (IP) assay (Supporting information, Supplementary Figure 3C). Importantly, PMEPA1β-PPNRm cannot

decrease p21 protein expression in PC-3 compared with the WT PMEPA1β (Figure 3B). These results imply that PMEPA1 possibly suppresses p21 expression by interacting with Smad2/3.

Consistent with the low level of p21 protein, PMEPA1β-overexpressing PC-3 has more cells in the S phase and fewer cells in the G1 phase compared with the LacZ-, PMEPA1β-PPNRm-, and PMEPA1α-overexpressing cells (Figure 3C). Furthermore, PMEPA1β-overexpressing PC-3 cells proliferate faster than other populations in the course of 6 days, as determined by the sulphorhodamine B (SRB) assay (Figure 3D).

PMEPA1 suppresses p21 through Smad3/4 but not Smad2

Since the SIM of PMEPA1 is required to suppress p21 expression in PC-3 (Figure 3B) and PMEPA1 interacts with Smad2/3 via the SIM and suppresses the Smad2/3

activation by TGF β [15], PMEPA1 may down-regulate p21 through blocking the TGF β –Smads signaling pathway. To test this, we depleted PMEPA1 in PC-3 and treated the cells with TGF β . Consistent with previous reports [7,14,15], PMEPA1 expression was induced by TGF β in PC-3 (Figure 4A). The induction of PMEPA1 by TGF β can be blocked by depletion of Smad3 or Smad4 but not Smad2 in PC-3 (Supporting information, Supplementary Figure 4). Importantly, knockdown of PMEPA1 additively increased the TGF β -induced p21 expression in PC-3 (Figure 4A). Consistently, PMEPA1 depletion additively suppressed the TGF β -induced PC-3 growth arrest, as determined by DNA synthesis (Figure 4A). These observations clearly indicate that endogenous PMEPA1 is induced by TGF β to negative feedback control the TGF β response.

To test whether PMEPA1 indeed down-regulates p21 through Smads, we knocked down Smad2, Smad3, and Smad4 in PC-3, respectively. As shown in Figure 4B, knockdown of Smad3, but not Smad2, down-regulated p21 and rescued the PMEPA1si-induced p21 up-regulation. As expected, knockdown of Smad4 showed the same rescue effect as knockdown of Smad3 (Figure 4B). Interestingly, PMEPA1 depletion decreased the protein expression levels of c-Myc, a well-known TGF β –Smad3 target that inhibits *p21* gene transcription [12]. Consistently, knockdown of either Smad3 or Smad4 increased the c-Myc expression levels and rescued the PMEPA1si-induced c-Myc down-regulation. These results suggest that the Smad3/4 complex may be essential for endogenous PMEPA1 to suppress p21 expression through c-Myc.

It has been suggested that PMEPA1 inhibits Smad3 phosphorylation in response to TGF β . Indeed, we found that PMEPA1 depletion modestly increased the pSmad3 level in PC-3 after TGF β treatment for 1 h. In addition, overexpression of PMEPA1 β showed the opposite effect (Figure 4C). The pSmad3 level cannot be detected without exogenous TGF β . However, the c-Myc and p21 expression changes initiated by PMEPA1 manipulation in PC-3 are independent of exogenous TGF β . It seems that PMEPA1 suppresses the basal level of Smad3 activity.

PMEPA1 is a cytoplasmic membrane protein, based on immunofluorescence staining (Supporting information, Supplementary Figure 3B). The active Smad3 must be in the nucleus and form a transcription complex with Smad4. To test whether cytoplasmic PMEPA1 retains Smad3 and prevents it from nuclear translocation, we examined the localization of Smad3 in the absence and presence of TGF β . As shown in Figure 4D, the nuclear Smad3 level in PMEPA1 β -overexpressing PC-3 cells is obviously lower than that in LacZ-overexpressing PC-3 cells. Again, PMEPA1 β suppressed Smad3 nuclear accumulation independently of exogenous TGF β , although TGF β significantly increased the nuclear accumulation of Smad3 (Figure 4D).

PMEPA1 suppresses p21 expression partially through up-regulating c-Myc expression

To test whether PMEPA1 functions through c-Myc, we depleted PMEPA1 by two different siRNAs in PC-3 and found that the c-Myc protein and mRNA levels were down-regulated (Figure 5A). The protein degradation of c-Myc was not affected by PMEPA1 (Supporting information, Supplementary Figures 1C and 1D). Additionally, we overexpressed c-Myc in PMEPA1-depleted PC-3 cells to investigate whether it can block PMEPA1si-induced p21 up-regulation. As expected, c-Myc overexpression decreased the p21 protein level and partially rescued PMEPA1si-induced p21 up-regulation (Figure 5B). Furthermore, c-Myc depletion by two different siRNAs in PC-3 dramatically up-regulated the p21 protein levels, similar to PMEPA1si (Figure 5C). However, PMEPA1 depletion increased p21 more efficiently than c-Myc depletion, even though the c-Myc siRNAs depleted more c-Myc than the PMEPA1 siRNA (Figure 5C). Thus, it is very likely that PMEPA1 suppresses p21 expression partially through c-Myc.

To further test whether PMEPA1 inhibits p21 expression through the Smad3–c-Myc pathway, we compared the c-Myc protein levels in WT and mutant PMEPA1-overexpressed PC-3 cells and found that both PMEPA1 isoforms up-regulated the c-Myc protein levels compared with LacZ (Figure 5D). PMEPA1 β -PPNRM cannot increase c-Myc and decrease p21 protein levels, supporting the finding that Smad3 binding is required for c-Myc up-regulation and p21 down-regulation. When c-Myc was depleted, PMEPA1 β overexpression failed to suppress p21 expression in PC-3 (Figure 5D). These findings further suggest that PMEPA1 inhibits p21 expression at least partially through up-regulating c-Myc expression.

PMEPA1 depletion inhibits prostate cancer growth *in vivo*

To provide proof-of-concept evidence that inhibition of PMEPA1 can be used to suppress prostate cancer *in vivo*, we generated two PMEPA1 stable knockdown PC-3 populations using two different shRNAs. Consistent with the results in Figures 2 and 5, stable knockdown of PMEPA1 decreased the c-Myc protein levels, increased the p21 protein levels (Figure 6A), and suppressed DNA synthesis *in vitro* (Figure 6B). When the PC-3-PMEPA1sh (#2) and PC-3-Lucsh control cells were injected into the SHO male mice, the PMEPA1sh cells grew significantly more slowly than the Lucsh control cells (Figure 6C). The mean tumour weight from PMEPA1sh was about twice that from the Lucsh control after 70 days of growth *in vivo* (Figure 6D). These results suggest that PMEPA1 could be a potential therapeutic target for AR-negative prostate cancer.

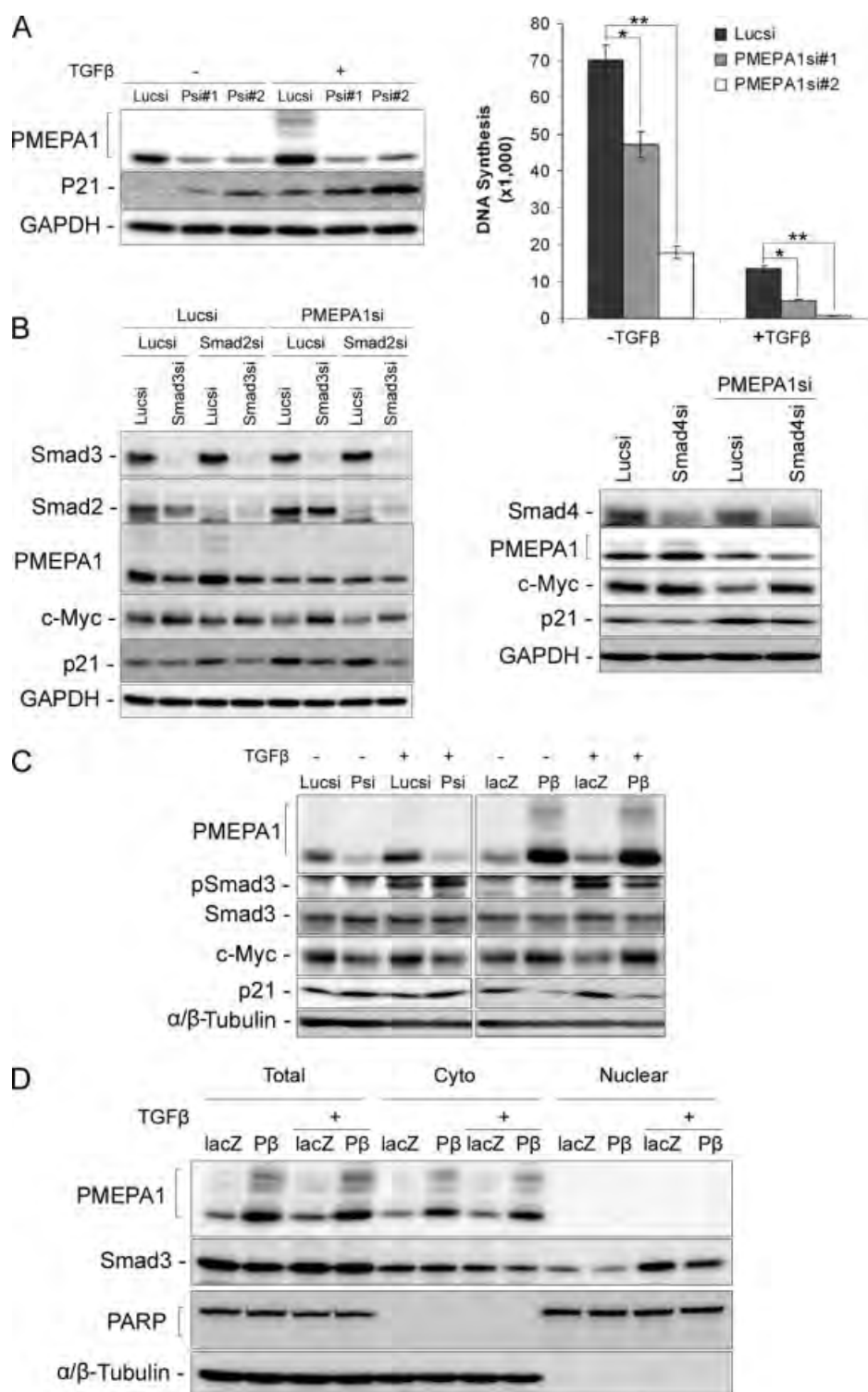


Figure 4. PMEPA1 suppresses p21 expression and cell proliferation through blocking Smad3 nuclear translocation in the presence and absence of TGFβ. (A) Knockdown of PMEPA1 increases the TGFβ-induced p21 expression in PC-3, as determined by immunoblotting (left panel). PC-3 cells were transfected with different PMEPA1 siRNAs. On the second day after transfection, the cells were serum-starved overnight and treated with 2 ng/ml TGFβ for 6 h. Knockdown of PMEPA1 increases the TGFβ-induced PC-3 growth arrest, as determined by the DNA synthesis assay (right panel). * $p < 0.05$; ** $p < 0.01$, t -test. (B) Knockdown of Smad3, but not Smad2, rescues PMEPA1 siRNA#1-induced c-Myc down-regulation and p21 up-regulation in PC-3 (left panel). Knockdown of Smad4 rescues PMEPA1 siRNA#1-induced c-Myc down-regulation and p21 up-regulation in PC-3 (right panel). (C) Knockdown of PMEPA1 increases and overexpression of PMEPA1β decreases Smad3 phosphorylation in the presence of TGFβ (2 ng/ml, 1 h) in PC-3. (D) Overexpression of PMEPA1β in PC-3 decreases the nuclear Smad3 protein levels in the absence and presence of TGFβ (2 ng/ml, 1 h). PARP is a nuclear marker; α/β-tubulin is a cytoplasmic marker.

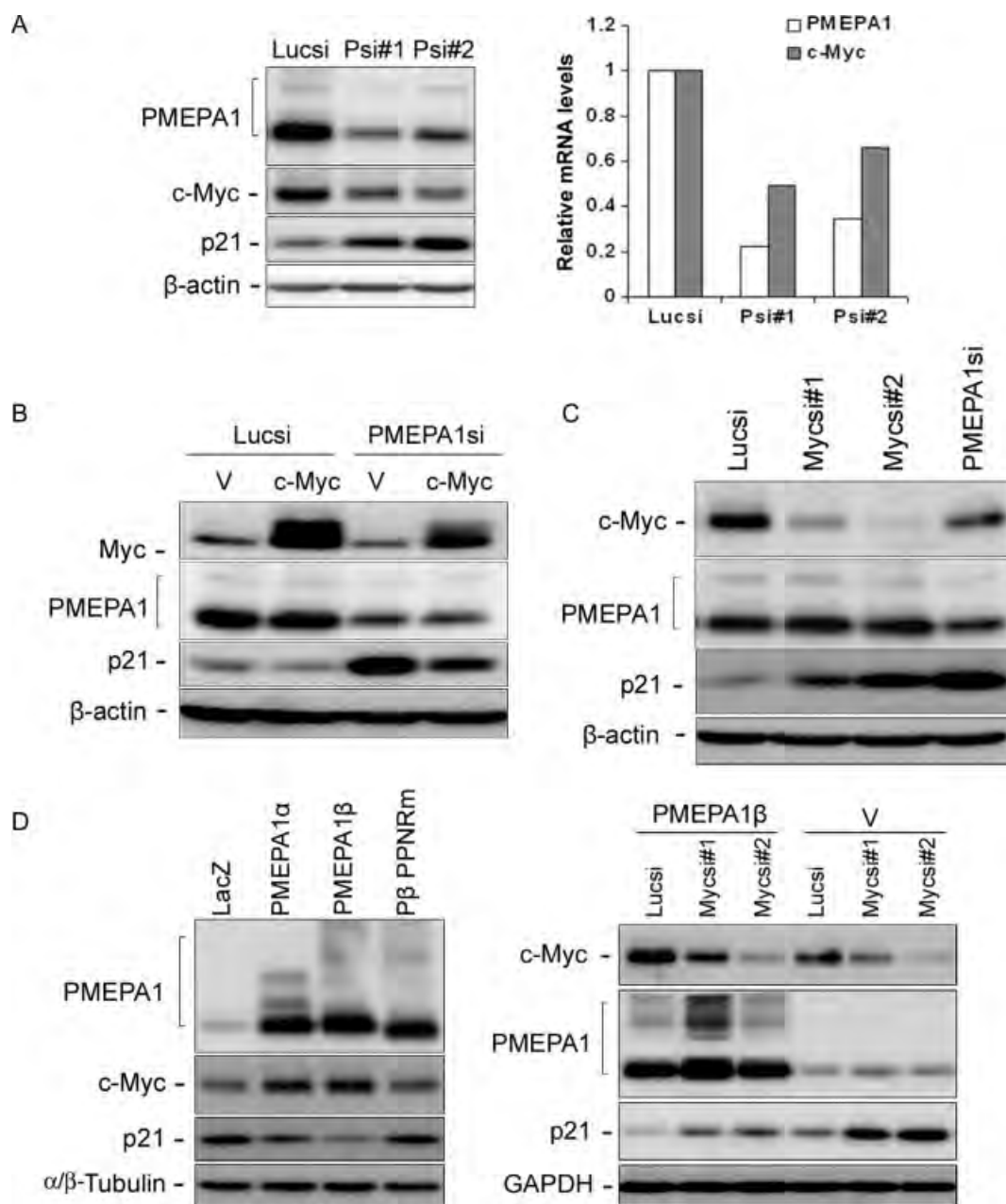


Figure 5. PMEPA1 suppresses p21 expression partially through up-regulating c-Myc expression. (A) Knockdown of PMEPA1 by two different siRNAs decreases c-Myc and increases the p21 protein expression levels in PC-3, as determined by immunoblotting (left panel). Knockdown of PMEPA1 decreases the c-Myc mRNA levels in PC-3, as determined by qRT-PCR (right panel). (B) Forced overexpression of c-Myc inhibits p21 protein expression and partially rescues the PMEPA1 siRNA#2-induced p21 protein expression increase in PC-3. PC-3 cells were transiently transfected with the siRNA and the c-Myc-expressing plasmid. (C) Knockdown of c-Myc is sufficient to down-regulate p21 protein expression in PC-3; however, it is not as efficient as knockdown of PMEPA1. (D) Forced overexpression of PMEPA1 up-regulates the c-Myc protein levels and inhibits the p21 protein levels in PC-3 in a PPNR motif-dependent manner. Knockdown of c-Myc by two different siRNAs rescues the PMEPA1 β overexpression-induced p21 protein expression decrease in PC-3.

The PMEPA1 protein is overexpressed in a subset of prostate cancers

PMEPA1 mRNA is overexpressed in multiple solid tumours, including breast and ovarian cancers [5], renal cell carcinoma [9], and colon cancer [7]. In contrast, the *PMEPA1* mRNA levels have been shown to be down-regulated in 64.5% (40/62) and up-regulated in 16.1% (10/62) of prostate tumours [18]. However, the PMEPA1 protein expression in prostate cancer has never been tested. We examined the PMEPA1

protein expression in seven prostate cell lines, including two immortalized cell lines (PZ-HPV7 and RWPE1), three AR-positive cancer cell lines (22Rv1, LAPC4, and LNCaP), and two AR-negative cancer cell lines (DU145 and PC-3). As shown in Figure 7A, two PMEPA1 protein isoforms (39 kD β and 36 kD α) were detected in all cell lines. Interestingly, PMEPA1 β was up-regulated in all prostate cancer cell lines except DU145, compared with the two immortalized prostate cell lines. PMEPA1 α was only

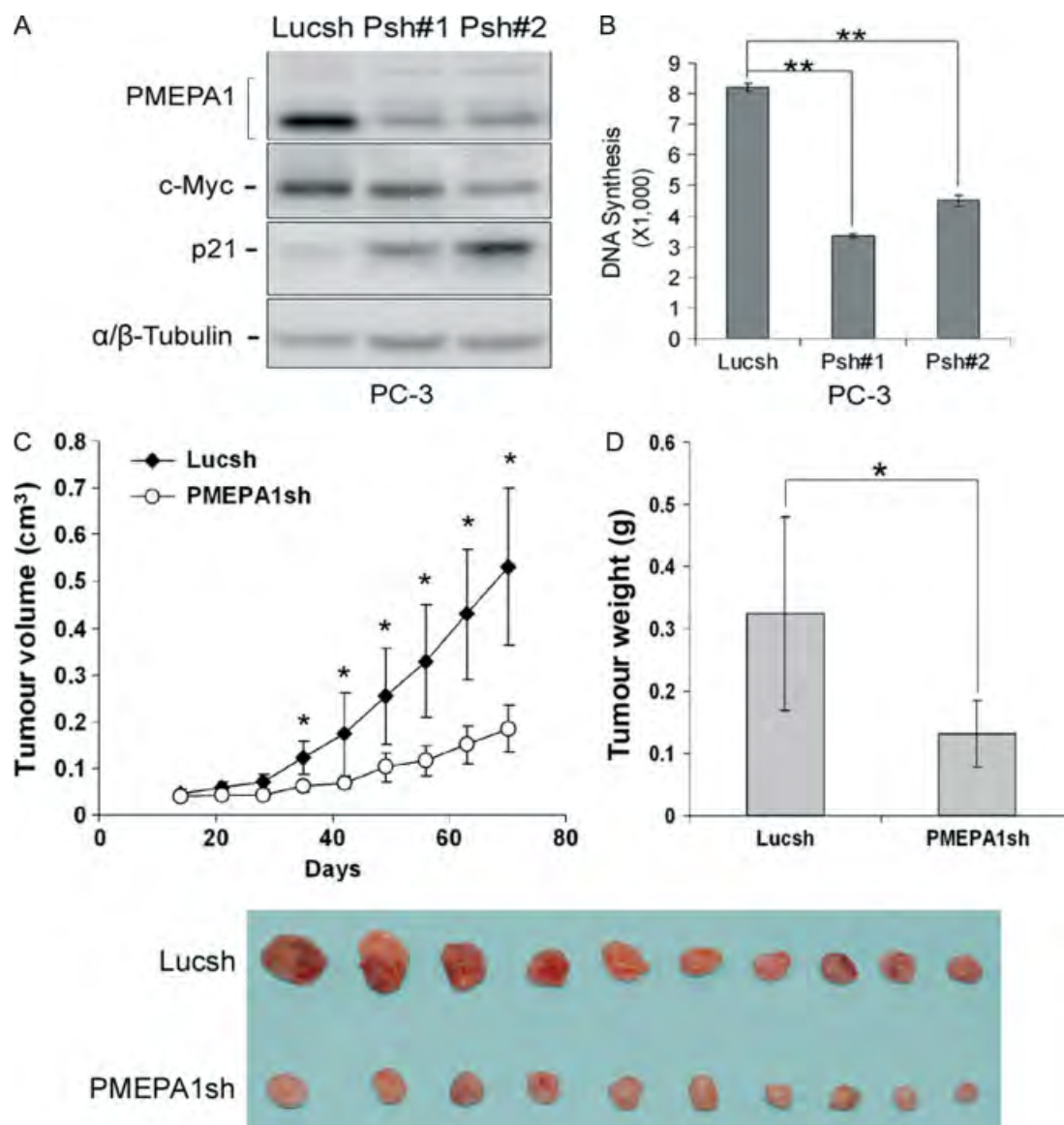


Figure 6. Depletion of PMEPA1 inhibits PC-3 prostate cancer growth *in vivo*. (A) Stable knockdown of PMEPA1 down-regulates c-Myc protein expression and up-regulates p21 protein expression in PC-3. (B) Stable knockdown of PMEPA1 in PC-3 significantly suppresses DNA synthesis *in vitro*. (C) The PC-3 PMEPA1sh#2 cell population grows more slowly than the Lucsh control cell population in male SHO mice. * $p < 0.05$ (t-test). (D) Stable knockdown of PMEPA1 significantly decreases the xenograft tumour weight ($n = 10$). * $p < 0.05$ (t-test). The tumours were harvested at day 70. Ten tumours from each group are shown.

up-regulated in PC-3 (Figure 7A). Thus, PMEPA1 β appears to be overexpressed in most prostate cancer cell lines.

To further confirm the overexpression of PMEPA1 in prostate cancer, we stained PMEPA1 in a tissue microarray that contained 93 prostate adenocarcinomas, ten normal prostates, and ten adjacent normal prostates by immunohistochemistry (IHC) staining. The PMEPA1 protein expression was detected in 30% of normal prostates, 50% of adjacent normal prostates, and 55% of prostate tumours (Table 1). The PMEPA1 protein expression was significantly ($p < 0.05$, Fisher's exact test) increased in grade 3 prostate tumours compared with normal prostates (Table 1 and Figure 7B). These results suggest that PMEPA1 is overexpressed in a subset of prostate cancers.

Discussion

PMEPA1 has been implicated in human cancers because of gene amplification, mRNA expression alterations, and induction by androgen and other growth factors. However, the roles and mechanistic actions of PMEPA1 in cancers have not been well characterized. Here, we have provided several lines of evidence to support that PMEPA1 promotes AR-negative prostate cell proliferation through suppressing the Smad3/4–c-Myc–p21 signaling pathway. First, the PMEPA1 β protein is overexpressed in most prostate cancer cell lines compared with the immortalized prostate cell lines (Figure 7A). The PMEPA1 protein is detected in 30% of normal prostate tissues but in 72% of grade 3 prostate adenocarcinomas (Figure 7B and Table 1). Second, loss-of-function and

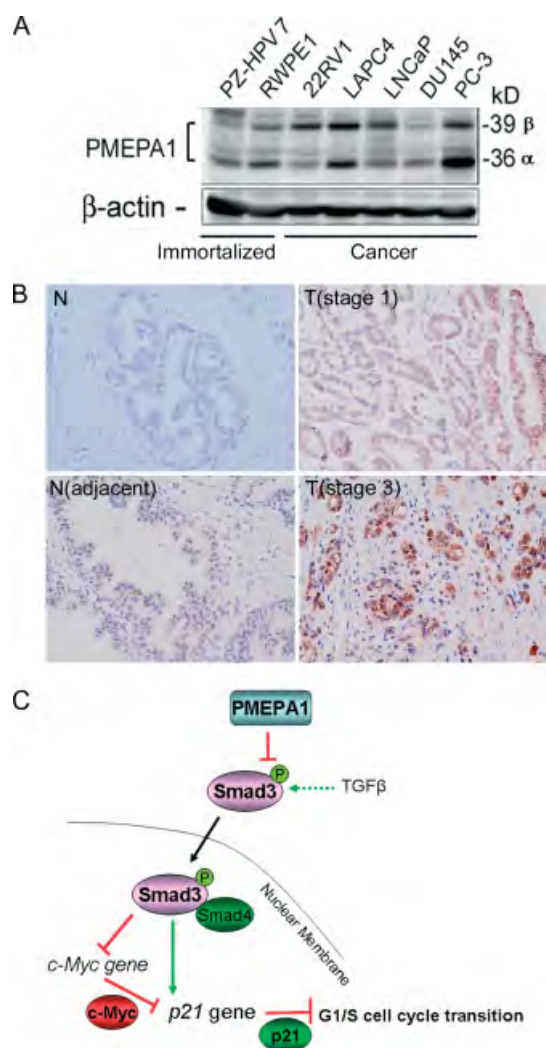


Figure 7. PMEPA1 is overexpressed in prostate cancer, and the pathway by which PMEPA1 inhibits cell proliferation. (A) Protein expression of PMEPA1, both 36 kD α and 39 kD β isoforms, in the immortalized prostate epithelial cell lines (PZ-HPV7 and RWPE1) and five prostate cancer cell lines was measured by immunoblotting. β -actin served as the loading control. (B) Examples of IHC staining for PMEPA1 in normal prostate (N; score 0), adjacent normal (score 3), and prostate carcinomas (T; stage 1: score 6 and stage 3: score 8). (C) PMEPA1 is a TGF β -induced target gene overexpressed in prostate cancers. The PMEPA1 protein interacts with Smad3 and inhibits its phosphorylation and nuclear translocation induced by TGF β . PMEPA1 also inhibits Smad3 nuclear translocation without exogenous TGF β in PC-3. The nuclear Smad3 and Smad4 form active transcriptional complexes that inhibit *c-Myc* and promote *p21* gene transcription. Smad3/4 and *c-Myc* synergistically regulate the *p21* gene transcription. As a cyclin-dependent kinase inhibitor, *p21* suppresses G1/S cell cycle transition, DNA replication, and cell proliferation.

gain-of function of PMEPA1 in AR-negative PC-3 cells inhibit and promote cell cycle progression and cell proliferation through oppositely regulating p21 expression (Figures 2–4). Following that, we demonstrated that the regulation of p21 by PMEPA1 is mediated by Smad3/4 and their downstream target *c-Myc* (Figures 4 and 5). Additionally, PMEPA1 is induced by TGF β as a negative feedback loop to control the TGF β signaling pathway (Figure 4). Finally, depletion of PMEPA1 inhibits PC-3 xenograft growth in mice

(Figure 6). The role and functional action of PMEPA1 are summarized in a signaling pathway model of PMEPA1–Smad3/4–*c-Myc*–p21 (Figure 7C).

PMEPA1 may be an oncogene in a subset of prostate cancer. First, the PMEPA1 protein is significantly increased in most prostate cancer cell lines and grade 3 prostate tumours. Second, PMEPA1 depletion causes cell growth arrest through down-regulating *c-Myc* and up-regulating p21. Additionally, overexpression of PMEPA1 β promotes cell cycle through up-regulating *c-Myc* and down-regulating p21. *c-Myc* is a very important oncogene for prostate cancer progression. Gene amplification of *c-Myc* has been associated with androgen-independent prostate cancer [24,25]. *c-Myc* overexpression can induce androgen-independent growth [26]. In addition to the *in vitro* results, we confirmed that PMEPA1 inhibition suppresses tumour growth *in vivo*. Our conclusions are supported by several previous studies. The *PMEPA1* gene locus is frequently amplified in prostate cancer [1]. *PMEPA1* mRNA is overexpressed in a variety of solid tumours, including a prostate xenograft and a subset of prostate tumours [4–6,18]. Consistently, *PMEPA1* mRNA expression is induced by growth factors in the cell cycle S phase [5]. The oncogenic role of PMEPA1 does not seem to be limited to prostate cancer because it also suppresses p21 expression in the HaCaT human keratinocyte cell line [15] and the HeLa cervical cancer cell line (Supporting information, Supplementary Figure 1A).

In contrast, PMEPA1 has been suggested to be a tumour suppressor in prostate cancer. First, the *PMEPA1* mRNA levels have been shown to be down-regulated in 64.5% (40/62) of prostate tumours [18]. Second, PMEPA1 is induced by androgen as a negative feedback loop to suppress the androgen signaling through targeting AR for degradation [19]. Li *et al* showed that PMEPA1 depletion increases the cell number in the S phase in the AR-positive LNCaP prostate cancer cell line [19]. We found that depletion of PMEPA1 in LNCaP and 22Rv1 prostate cancer cell lines does not induce p21 (data not shown). It seems that only the proliferation of AR-negative prostate cancers with intact TGF β signaling depends on PMEPA1. Whether the PMEPA1 protein expression in prostate tumours is limited to AR-negative tumours and can be used as a diagnostic and prognostic biomarker should be further studied in more clinical samples with pathological parameters.

PMEPA1 plays an important role in the TGF β pathway. First, PMEPA1 is induced by TGF β in PC-3 through Smad3/4 (Figure 4A and Supporting information, Supplementary Figure 4). Second, PMEPA1 depletion increases the TGF β -induced p21 and cell growth arrest in PC-3 (Figure 4A). Similar results were observed in TGF β -sensitive HaCaT and HeLa cells (data not shown). Consistent with a previous study [15], PMEPA1 uses its SIM domain to interact with Smad2/3 and suppresses Smad3 phosphorylation and nuclear translocation in response to TGF β (Figures 3

and 4). Interestingly, Smad3 is exclusively used by PMEPA1 to regulate c-Myc and p21 transcription in PC-3, while Smad2 plays no role. Besides c-Myc and p21, PMEPA1 also regulates other TGF β target genes, such as p27 (Figure 2), p15^{Ink4b}, Jun B, and PAI1 [15] in HaCaT. Depletion of p21, but not p27, completely rescued the PMEPA1 siRNA-induced growth arrest in both RWPE1 and PC-3 (Figure 2), suggesting that p21 mediates the PMEPA1 pro-proliferation functions in prostate cells. It is noteworthy that regulation of c-Myc and p21 by PMEPA1 is independent of exogenous TGF β . It is possible that PMEPA1 suppresses Smad3 nuclear translocation without TGF β or in the presence of low levels of TGF β , although Smad3 phosphorylation cannot be detected without exogenous TGF β (Figure 4). Nevertheless, PMEPA1 can be induced by TGF β as a negative feedback loop to directly control the Smad3 activation. Thus, our work illustrates the exact signaling pathway by which PMEPA1 promotes the cell cycle, although a similar role of PMEPA1/TMEPAI in the TGF β pathway has been recently demonstrated in other cancers [15]. Our independent study will not only help to clarify the role of PMEPA1 in prostate cancer, but also help to extend the oncogenic role of PMEPA1 *in vivo*.

Two human PMEPA1 isoforms (α and β) can be detected in prostate cell lines. PMEPA1 β and PMEPA1 α differ in their N-terminuses. PMEPA1 β has a longer fragment (35-aa residues). Although both isoforms have the SIM, PY motifs, and TM domain, and inhibit Smad2 phosphorylation with a similar efficiency [15], their functions of regulating p21 are different. PMEPA1 β is significantly more potent than PMEPA1 α in suppressing p21 expression (Figures 3B and 5D). As a result, the PMEPA1 α variant does not significantly promote cell cycle progression and cell growth as the β variant does (Figure 3). The β -isoform-specific N-terminal fragment may play a key role in the suppression of p21 expression. There are putative myristoylation and glycosylation sites in the β -isoform-specific N-terminal fragment [5]. Interestingly, the PMEPA1 β variant is distributed more diffusely than PMEPA1 α , which concentrates around the nucleus (Supporting information, Supplementary Figure 3B). The structure, modifications, and functions of the PMEPA1 isoforms need further investigation in the future.

In summary, we have demonstrated that PMEPA1 is overexpressed in a subset of prostate cancers and promotes the cell cycle and cell proliferation through inhibiting the expression of p21 via the Smad3/4–c-Myc signaling pathway (Figure 7C). Furthermore, we have shown that PMEPA1 is induced by TGF β as a negative feedback loop to directly control Smad3 activation. Finally, suppression of PMEPA1 inhibits xenograft tumour growth. Thus, PMEPA1 might be developed as a molecular target for cancer prevention, diagnosis, and therapy in prostate cancer and other cancers.

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Author contribution statement

RL designed and performed experiments and wrote the draft manuscript. ZZ assisted in the experiments. JH performed IHC in prostate tumours. CC designed experiments and revised the paper.

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Note: References 27–29 are cited in the Supporting information to this article.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Supplementary materials and methods.

Figure S1. PMEPA1 does not alter *p21* mRNA and protein stability.

Figure S2. Knockdown of endogenous PMEPA1 inhibits prostate cell proliferation through up-regulating p21 expression.

Figure S3. PMEPA1 β suppresses p21 expression.

Figure S4. The induction of PMEPA1 by TGF β depends on Smad3/4, but not Smad2, in PC-3.

Table S1. The siRNA target sequences used in this study.

Table S2. The PCR primer sequences used in this study.

The Induction of KLF5 Transcription Factor by Progesterone Contributes to Progesterone-Induced Breast Cancer Cell Proliferation and Dedifferentiation

Rong Liu, Zhongmei Zhou, Dong Zhao, and Ceshi Chen

The Center for Cell Biology and Cancer Research (R.L., Z.Z., D.Z., C.C.), MS355, Albany Medical College, Albany, New York 12208; Key Laboratory of Animal Models and Human Disease Mechanisms (Z.Z., C.C.), Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Kunming, Yunnan, 650223, China

Progesterone (Pg) promotes normal breast development during pregnancy and lactation and increases the risk of developing basal-type invasive breast cancer. However, the mechanism of action of Pg has not been fully understood. In this study, we demonstrate that the mRNA and protein expression of Klf5, a pro-proliferation transcription factor in breast cancer, was dramatically up-regulated in mouse pregnant and lactating mammary glands. Pg, but not estrogen and prolactin, induced the expression of Krüppel-like factor 5 (KLF5) in multiple Pg receptor (PR)-positive breast cancer cell lines. Pg induced the KLF5 transcription through PR in the PR-positive T47D breast cancer cells. Pg-activated PR increased the KLF5 promoter activity likely through binding to a Pg response element at the KLF5 promoter. Importantly, Pg failed to promote T47D cell proliferation when the KLF5 induction was blocked by small interfering RNA. KLF5 is essential for Pg to up-regulate the expression of cell cycle genes, including *CyclinA*, *Cdt1*, and *E2F3*. In addition, KLF5 overexpression was sufficient to induce the cytokeratin 5 (CK5) expression, and the induction of CK5 by Pg was significantly reduced by KLF5 small interfering RNA. Consistently, the expression of KLF5 was positively correlated with that of CK5 in a panel of breast cancer cell lines. Taken together, we conclude that KLF5 is a Pg-induced gene that contributes to Pg-mediated breast epithelial cell proliferation and dedifferentiation. (*Molecular Endocrinology* 25: 0000–0000, 2011)

NURSA Molecule Pages: Nuclear Receptors: PR; Ligands: Progesterone | RU486 | MPA.

Progesterone (Pg) is essential for normal postnatal breast development during pregnancy and lactation by stimulating ductal side branching and development of lobuloalveolar structures (1). Under the stimulation of Pg, the mammary gland epithelium undergoes extensive proliferation and remodeling (1). Pg functions primarily through the ligand-activated nuclear receptor transcription factor Pg receptor (PR). PR knockout mice showed incomplete mammary gland ductal side branching and failure of lobuloalveolar structure development due to insufficient cell proliferation (2). However, the molecular mechanism of action of Pg and PR has not been completely elucidated.

Accumulated evidence suggests that Pg and PR promote mammary tumorigenesis (3, 4). Pg has been shown to stimulate breast cancer for menopausal women in several large-scale hormone-replacement therapy clinical studies (5–7). In these studies, Pg plus estrogen significantly increased the risk of invasive breast cancer compared with estrogen alone. Additionally, progestins have been shown to have proliferative effects in the PR-positive breast cancer cell lines *in vitro* (8) and in nude mice (9). Furthermore, progestins increased mammary tumor incidence in rats (10–12) and dogs (13).

Recently, progestins have been shown to reprogram a subset of estrogen receptor (ER)+PR+cytokeratin 5

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Abbreviations: ChIP, Chromatin immunoprecipitation; CK5, cytokeratin 5; ER, estrogen receptor; ESC, embryonic stem cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; KLF5, Krüppel-like factor 5; KO, knockout; MPA, medroxyprogesterone 17-acetate; Pg, progesterone; PR, Pg receptor; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA.

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(CK5)– differentiated cells into ER-PR-CK5+ basal-like progenitor cancer cells *in vitro* and *in vivo* (14–16). The ER-PR-CK5+ cells have progenitor potential and are less prone to drug-induced apoptosis during the endocrine therapy and chemotherapies (16). Interestingly, Pg increased the development of CK5+ basal type breast cancer in rats (17). However, the mechanism by which progestins reprogram ER+PR+CK5– cells into ER-PR-CK5+ cells is not clear.

Krüppel-like factor 5 (KLF5) is a transcription factor that regulates cell proliferation, survival, differentiation, and embryonic stem cell (ESC) self-renewal. Previously, we demonstrated that KLF5 overexpression promotes the G₁/S cell cycle progression (18) and breast cell proliferation, survival, and tumor growth (19, 20). Furthermore, KLF5 has been reported to regulate smooth muscle cell and adipocyte differentiation (21, 22). Most recently, KLF5 was shown to promote the self-renewal of ESC and to maintain ESC in an undifferentiated state (23, 24). Interestingly, the gene expression signature of basal-like breast cancer cells is similar to that of ESC as shown through analyzing the microarray data from 1211 breast tumors (25). KLF5 and eight other genes are highly expressed in basal-type breast tumors (25). Indeed, positive *KLF5* mRNA expression is an unfavorable prognostic marker correlated with shorter survival for breast cancer patients (26). However, the transcriptional regulation of **KLF5** in breast cancer is largely unknown. We hypothesize that *KLF5* is a Pg and PR downstream target gene and plays an important role in Pg-induced breast cancer cell proliferation and dedifferentiation.

KLF5 should be italicized

Results

The expression of Klf5 is increased in mouse mammary glands during pregnancy and lactation stages

To explore the physiological regulation of Klf5 in the breast, we first examined the Klf5 protein expression by immunoblotting in the mouse mammary glands during four different stages (virgin, pregnancy, lactation, and involution). As shown in Fig. 1A, the protein expression level of Klf5 was undetectable in mammary glands during the stages of virgin and involution but increased dramatically in mammary glands during the stages of pregnancy and lactation. The expression up-regulation of *Klf5* during the stages of pregnancy and lactation occurred at the mRNA level as assessed by RT-PCR (Fig. 1B). These results imply that the *Klf5* transcription may be regulated by a specific hormone produced during the stages of pregnancy and lactation.

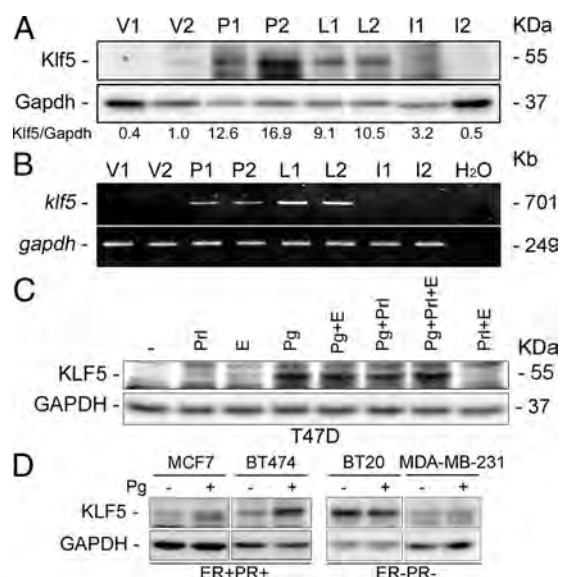


FIG. 1. The KLF5 expression is induced in mouse mammary glands during the stages of pregnancy and lactation and in PR-positive breast cancer cell lines by Pg. A, The protein expression levels of Klf5 are specifically up-regulated in mouse mammary glands during the stages of pregnancy and lactation. Mouse mammary glands were collected from female mice in different stages [V, virgin; P, pregnancy (13.5 d and 16.5 d); L, lactation; I, involution]. Two mice (B6) in each stage were examined. Mammary gland tissue lysates were used for immunoblotting. Gapdh was used as a loading control. B, The mRNA expression levels of *Klf5* are specifically up-regulated in mouse mammary glands during pregnancy and lactation. The *Klf5* mRNA levels were measured by RT-PCR. *Gapdh* was used as a loading control. C, Pg but not estrogen (E) or prolactin (Prl) induces the KLF5 protein expression. T47D cells were seeded in 12-well plates at 2.2×10^5 cells per well. The cells were serum-starved for 24 h and treated with Pg (100 nM), E (20 nM 17 β -estradiol), Prl (100 ng/ml), or different combinations for 8 h. The KLF5 protein expression was measured by immunoblotting. GAPDH serves as a loading control. D, Pg induces the KLF5 protein expression in PR-positive but not in PR-negative breast cancer cell lines. All cells were treated with 100 nM Pg for 8 h or left untreated in serum-free media. The data shown in this figure are representative of two independent experiments.

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Pg induces the KLF5 expression in ER+PR+ breast cancer cell lines

The postnatal development of normal mammary glands involves a coordinated effort of pituitary and ovarian hormones. Of those hormones, estrogen, Pg, and prolactin are the most significant regulators for duct growth and alveolar development. In an ER+PR+ luminal breast cancer cell line T47D in which the endogenous KLF5 protein level is very low, we demonstrated that Pg, but not estrogen and prolactin, induced the KLF5 protein expression (Fig. 1C). Estrogen and prolactin could not even increase the KLF5 expression when in combination with Pg (Fig. 1C). Pg also induced the KLF5 protein expression in several other ER+PR+ breast cancer cell lines, such as MCF7 and BT474, but not in ER–PR– breast cancer cell lines BT20 and MDA-MB-231 (Fig. 1D).

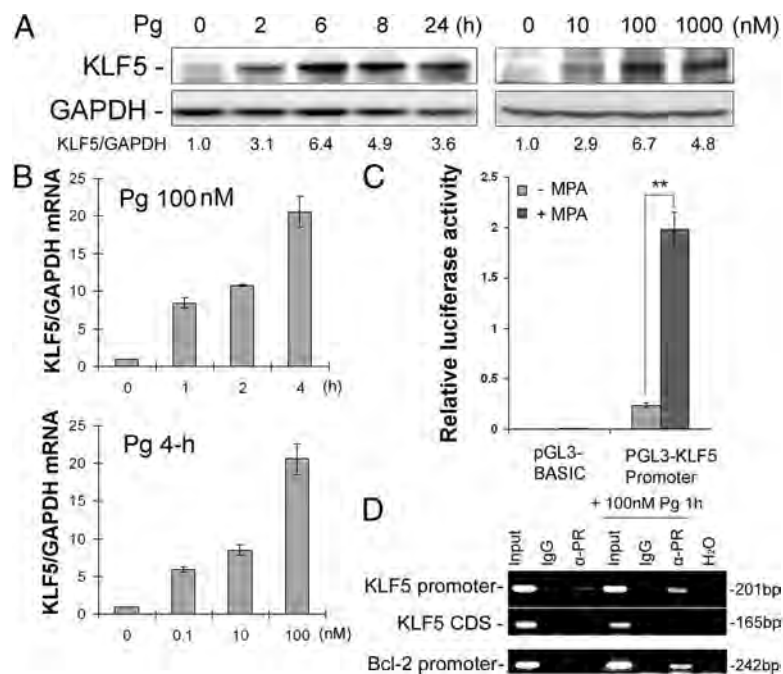


FIG. 2. Pg induces the KLF5 expression in time- and dosage-dependent manners and activates the KLF5 transcription in T47D. **A**, The KLF5 protein was induced by Pg in a time- and dosage-dependent manner. T47D cells were treated with Pg at 100 nM for the indicated time (*left panel*) or 8 h at the indicated concentration (*right panel*). **B**, Pg induced the KLF5 mRNA expression in a time- and dosage-dependent manner. The KLF5 mRNA expression levels were measured by qRT-PCR. Error bars show SD values from triplicates in a single representative experiment. **C**, Pg activated the KLF5 promoter. T47D cells were transfected with PR-B expressing plasmid and the KLF5 promoter luciferase reporter. One day after transfection, the cells were serum-starved overnight and treated with 20 μ M MPA for 24 h. Cell lysates were collected for the dual-luciferase reporter assay. **, $P < 0.001$. **D**, PR specifically binds to the KLF5 promoter *in vivo* as determined by ChIP assays. The input DNA and water were used as the positive and negative controls. The coding sequence (CDS) of KLF5 was amplified to test the binding specificity. The Bcl-2 gene promoter region was amplified as a positive control for ChIP (28). All data shown in this figure have been confirmed by independent repeated experiments.

Pg activates the KLF5 transcription

Then, a time- and dosage-course experiment was conducted in T47D. As shown in Fig. 2, A and B, KLF5 was induced by Pg in time- and dosage-dependent manners at both the protein and mRNA levels. The induction of KLF5 by Pg could be detected after 1 h, suggesting that KLF5 is a Pg early-responsive gene and a possible PR direct-target gene. The maximum induction time for the KLF5 protein is about 6 h (Fig. 2A). The KLF5 expression can be significantly induced by Pg at physiological concentrations (1–100 nM). Similar results were observed in MCF7 (data not shown). These results suggest that Pg may increase the KLF5 transcription. To test whether Pg can activate the KLF5 promoter, we performed a luciferase reporter assay in T47D cells. As expected, the KLF5 promoter was significantly activated by synthetic Pg medroxyprogesterone 17-acetate (MPA) (Fig. 2C).

Because Pg activated the KLF5 gene promoter, we searched the KLF5 promoter sequence and identified a

6-bp potential Pg response element sequence (–548 TGTACA –543) (27). To determine whether PR binds to this Pg response element at the KLF5 promoter, we performed chromatin immunoprecipitation (ChIP) assays in T47D cells using the Bcl2 gene promoter as the positive control (28). As shown in Fig. 2D, the anti-PR antibody, but not the IgG control, specifically immunoprecipitated the KLF5 promoter rather than coding region. Furthermore, Pg dramatically enhanced the binding of PR to the KLF5 and Bcl2 promoters. These results suggest that KLF5 is a direct PR target gene.

Pg induces the KLF5 expression through PR

To test whether Pg induces the KLF5 expression through PR, we first blocked the PR activity using the PR antagonist RU-486 in T47D and found that the induction of KLF5 protein by Pg was completely abolished (Fig. 3A). Furthermore, two different PR small interfering (siRNA) almost completely blocked the KLF5 protein induction in T47D (Fig. 3B). These results clearly indicate that PR is required for the KLF5 induction by Pg.

The induction of KLF5 is essential for Pg to promote cell proliferation and gene expression

Because both Pg and KLF5 have been shown to promote cell proliferation, we wondered whether KLF5 is induced by Pg to promote cell proliferation. To test this, we blocked the Pg-induced KLF5 by a well-characterized KLF5 siRNA (18) and two PR siRNA in T47D and examined the DNA synthesis. As expected, Pg dramatically increased the DNA synthesis in Lucsi transfected cells (Fig. 3D). PR depletion not only blocked the KLF5 induction but also blocked the Pg-induced DNA synthesis increase (Fig. 3, C and D). When the KLF5 induction was depleted by KLF5 siRNA, Pg failed to increase the DNA synthesis in T47D (Fig. 3, C and D). To understand the mechanism by which KLF5 promoted cell proliferation, we examined the mRNA expression of several genes involved in cell cycle (29). As shown in Fig. 3E, the induction of *cyclinA*, *Cdt1*, and *E2F3* by Pg was blocked by the depletion of KLF5 or PR. These results suggest that the KLF5 induction is essential for Pg to stimulate gene expression and cell proliferation *in vitro*.

KLF5 contributes to Pg-induced CK5 expression

Horwitz and co-workers (14, 15) showed that progesterins could reprogram a small subset of ER+PR+CK5–

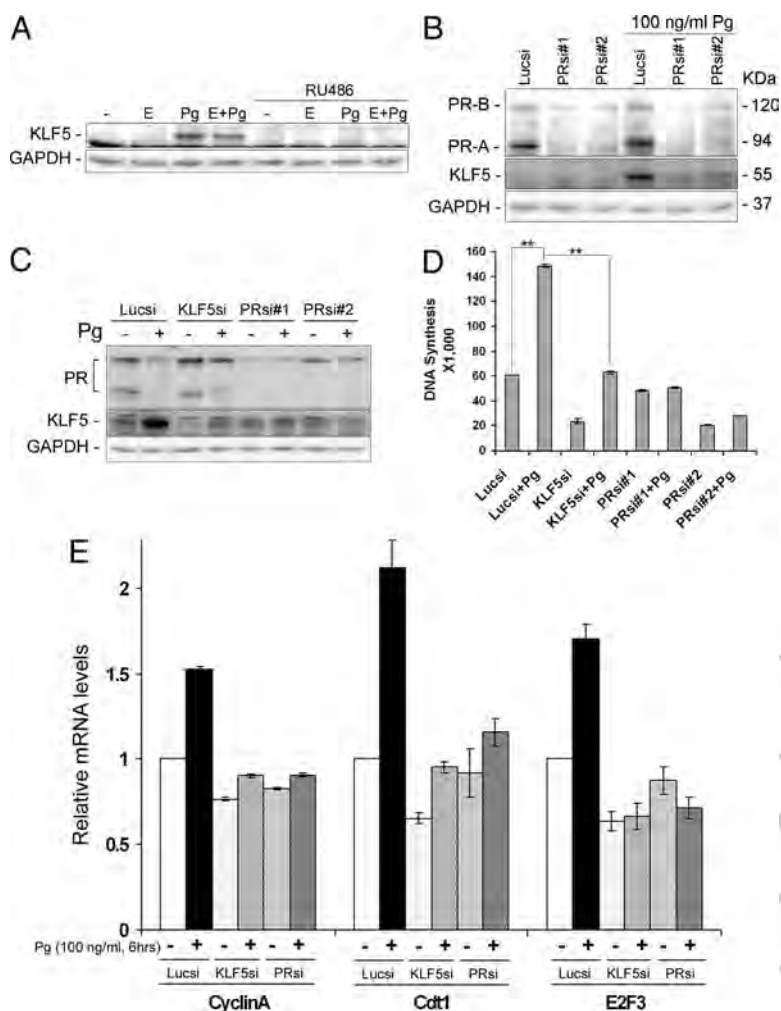


FIG. 3. The induction of KLF5 by Pg is through PR and is essential for Pg to promote T47D cell proliferation and gene expression. A, The Pg antagonist RU-486 (100 nM) completely blocked the KLF5 induction by Pg (100 nM) or by E (20 nM) + Pg (100 nM). T47D cells were treated with hormones and RU-486 for 8 h. B, Two different PR siRNAs blocked the induction of KLF5 by Pg. T47D cells were transfected with control Luciferase siRNA (Lucsi) or two different PR siRNAs (PRsi#1 and PRsi#2). One day after transfection, the cells were serum starved for 24 h and then were treated with Pg (100 nM) overnight. There are two PR isoforms (B and A). C, The KLF5 and PR siRNA silenced the KLF5 and PR protein expression in T47D in the absence and presence of Pg. D, KLF5 or PR depletion by siRNA significantly blocked the DNA synthesis increase induced by Pg in T47D. The DNA synthesis was measured by [³H]thymidine incorporation assay. KLF5 siRNA itself decreased the DNA synthesis due to the depletion of endogenous KLF5 expression. **, $P < 0.001$, t test. E, KLF5 or PR depletion by siRNA significantly blocked the Pg-induced expression of *CyclinA*, *Cdt1*, and *E2F3* in T47D. The mRNA levels were measured by qRT-PCR. GAPDH was used to normalize the input cDNA. Figures show representative data of three independent experiments.

T47D cells into ER-PR-CK5+ progenitor cells. As an ESC transcription factor, KLF5 may contribute to this dedifferentiation step. It has been shown that KLF5 is highly expressed in basal-type (CK5+) breast cancers (25). To test if KLF5 induces the CK5 expression, we infected T47D cells with green fluorescent protein (GFP) control and KLF5-IRES-GFP adenoviruses. Indeed, KLF5 induced the CK5 protein expression compared with the GFP control by immunofluorescence staining (IF) (Fig.

4A). However, only a small percentage (~2–3%) of T47D cells infected with KLF5 adenoviruses expressed CK5. Furthermore, the CK5 expression was not only limited to GFP/KLF5-positive cells. The ER and PR levels were not steadily down-regulated by KLF5 by immunoblotting (data not shown). A 10-fold induction of CK5 mRNA expression by KLF5 was detected by qRT-PCR in T47D (Fig. 4B).

Because Pg has been shown to induce the CK5 expression in a small subset of T47D cells (14, 15), we tested whether Pg induces CK5 through KLF5. Indeed, when the induction of KLF5 was blocked by KLF5 siRNA, Pg-induced CK5 mRNA expression was significantly but not completely blocked (Fig. 4C). The induction of *serum glucocorticoid regulated kinase* (SGK), a PR direct target gene (30), was not affected by KLF5 siRNA at all. As expected, PR siRNA blocked the induction of KLF5, CK5, and SGK by Pg. These findings suggest that KLF5 may contribute to Pg-mediated cell dedifferentiation as indicated by inducing the CK5 expression.

The expression of KLF5 is negatively correlated with ER/PR but positively correlated with CK5 in breast cancer cell lines

Progestins have been shown to suppress ER and PR expression in addition to inducing CK5 expression (14, 15). To further test if KLF5 contributes to the reduction of ER and PR by progestins, we treated T47D cells with MPA for 6 and 24 h and examined the KLF5, ER, and PR expression. With the induction of KLF5, MPA also dramatically decreased the ER and PR expression (Fig. 5A). However, when the induction of KLF5 was blocked by KLF5 siRNA, the reduction of ER and PR was not affected at all. These results imply that the MPA-induced loss of ER and PR expression is not mediated by KLF5.

Finally, we examined the expression of KLF5, ER, PR, and CK5 by immunoblotting in a panel of eight breast cancer cell lines (Fig. 5B). In agreement with our previous report (31), KLF5 is lowly expressed in all ER+CK5– and/or PR+CK5– breast cancer cell lines (MCF7, HCC1500, MDA-MB-134, and T47D) but highly expressed in ER-PR-CK5+ breast cancer cell lines (SW527, HCC1937, HCC1806, and SUM149). This expression pattern suggests that the KLF5 expression may be a signature of ER-PR-CK5+ cells.

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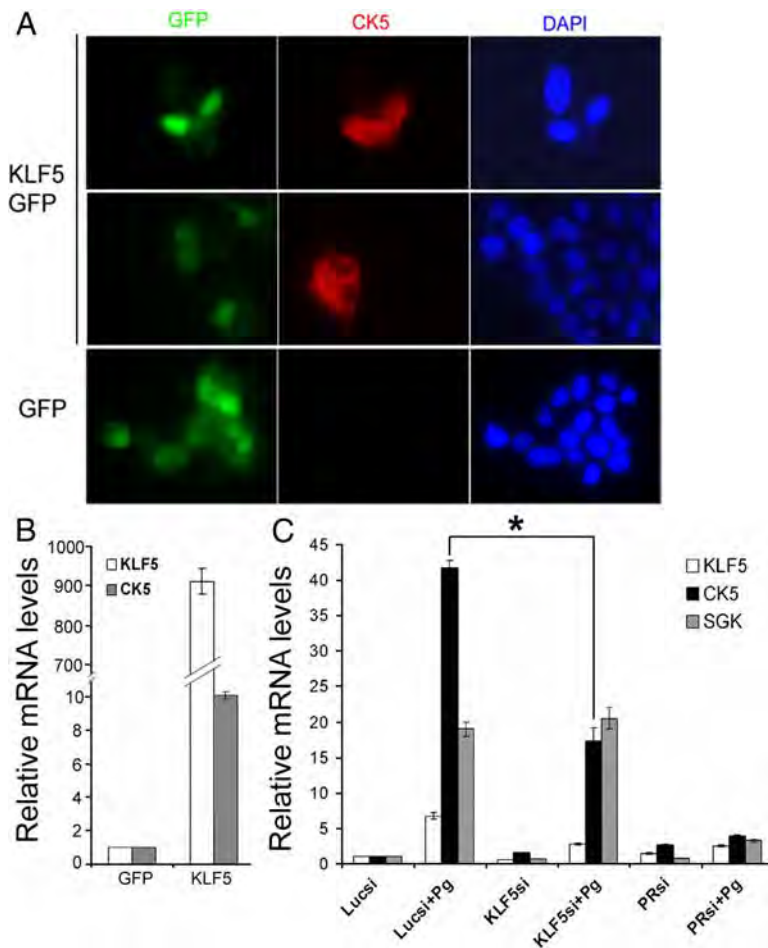


FIG. 4. KLF5 is sufficient to induce the CK5 expression and partially contributes to Pg-induced CK5 expression in T47D. A, KLF5 induces the CK5 protein expression in T47D, as determined by immunofluorescence. KLF5 was delivered into T47D by adenoviruses with the GFP marker (the GFP expression is independently regulated). The GFP adenoviruses were used as a negative control. B, KLF5 overexpression induced the CK5 mRNA expression. The *KLF5* and *CK5* mRNA levels were measured by qRT-PCR. C, KLF5 depletion significantly reduced the Pg-induced CK5 expression. T47D cells were transfected with Lucsi, KLF5si, and PRsi, respectively, and were treated with or without 100 nM Pg for 24 h. The *KLF5*, *CK5*, and *SGK* mRNA levels were measured by qRT-PCR. *, $P < 0.01$. Figures show representative data of three independent experiments. DAPI, 4',6-Diamidino-2-phenylindole.

Discussion

Pg and its cognate receptor PR are essential for normal breast development during pregnancy and regulate breast carcinogenesis. However, the mechanisms of the action of Pg and PR have not been fully understood. For the first time, we demonstrate that the KLF5 transcription factor is induced by Pg and PR and contributes to Pg-induced cell proliferation and CK5 expression in PR-positive breast cancer cells. Our findings may reveal a novel mechanism for Pg and PR to regulate the development of normal breast and breast cancer.

KLF5 is an important Pg/PR downstream gene promoting cell cycle. In PR-positive breast cancer cell lines, Pg induces quiescent cells to enter the cell cycle through up-regulating several G₁-S phase cell cycle proteins in-

cluding Cyclin D1 (32). KLF5 has also been shown to induce Cyclin D1 (33). However, the induction of cyclin D1 by progestins is not mediated by KLF5 (data not shown). The KLF5 direct target gene, fibroblast growth factor-BP, was also not induced by Pg in T47D (data not shown). We found that KLF5 is essential for Pg to induce the expression of cell cycle genes including *CyclinA*, *Cdt1*, and *E2F3* in T47D (Fig. 3E). These genes have been well implicated in Pg-induced breast cell cycle G₁/S transition (29).

Pg has been shown to reprogram a small subset of ER+PR+CK5– differentiated luminal cells into ER-PR-CK5+ progenitor cancer cells (14, 15). Given the significant role of KLF5 in maintaining ESC self-renewal and preventing their differentiation, it is not surprising that KLF5 contributes to Pg-initiated cell reprogram. KLF5 contributes to Pg-induced cell dedifferentiation as suggested by inducing the CK5 expression (Fig. 4). Interestingly, the induction of CK5 by KLF5 in T47D showed a paracrine manner. The KLF5-negative cells next to KLF5-positive cells also expressed CK5. It is possible that KLF5 induced the expression of yet to be identified secreted proteins that indirectly induced the CK5 expression. Our result is consistent with a previous report that Pg acts by a paracrine mechanism in mammary epithelial cells (34). In T47D cells, Pg only induced CK5 in a very small percentage of cells by IF (data not shown; we could not steadily detect the induced CK5 by immunoblotting). When the induction of KLF5 is blocked, the Pg-induced CK5 mRNA levels were significantly reduced. Thus, KLF5 is not only sufficient

to induce the CK5 expression but also essential for Pg to efficiently induce the CK5 expression.

The induction of KLF5 by progestins was also in parallel with the reduction of ER/PR in T47D. This explains the observation that KLF5 is highly expressed in ER-PR-CK5+ basal-like breast cancer cells although KLF5 is induced by Pg in ER+PR+CK5– luminal breast cancer cells. Thus, Pg can reprogram the ER+PR+KLF5-CK5– breast cancer cells into the ER-PR-KLF5+CK5+ cells by inducing the expression of *KLF5*, *CK5*, and other genes. How the KLF5 expression is maintained in ER-PR– breast cancer cells is still unknown. Recently, KLF5 has been reported to interact with ER α and suppresses its functions (35). However, KLF5 is not responsible for the loss of ER/PR after progestin stimulation. Whether KLF5

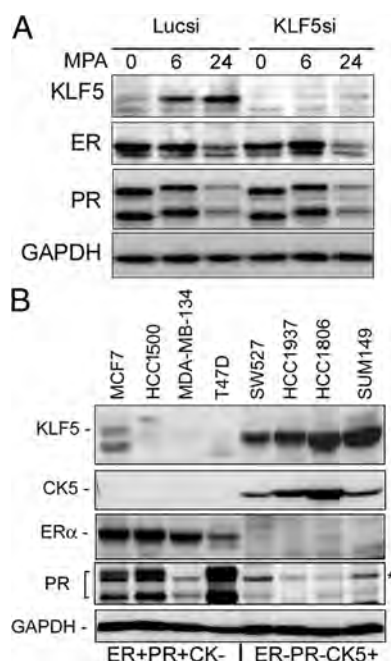


FIG. 5. The expression of KLF5 is negatively correlated with ER/PR but positively correlated with CK5 in breast cancer cell lines. A, The induction of KLF5 by MPA (20 μ M) was in parallel with the reduction of ER and PR in T47D. KLF5 depletion did not block the Pg-induced loss of ER and PR expression. B, The protein expression of KLF5, CK5, ER, and PR in eight breast cancer cell lines was measured by immunoblotting. GAPDH was used as a loading control. Asterisk indicates a nonspecific band. Data show representative result of three independent experiments.

contributes to progesterin-induced other differentiation gene expression is unknown. Because ER-PR-CK5+ cells from luminal breast cancers are less prone to drug-induced apoptosis and resistant to standard endocrine and chemotherapies (16), Pg/PR/KLF5 targeted therapy may prevent ER/PR-positive breast cancer progression and overcome the drug resistance.

Accumulated evidence suggests that KLF5 is a pro-proliferation and pro-survival oncogenic transcription factor specifically overexpressed in ER-PR-CK5+ breast cancers (19, 20, 31). The transcription of KLF5 has been shown to be up-regulated by several oncogenes including *Ras* (36), *ErbB2* (37), and *Wnt1* (38). *We demonstrated that Pg induces the KLF5* transcription through PR (Fig. 3). Interestingly, KLF5 is also an androgen-induced gene in androgen receptor-positive prostate cancer cell lines (27, 39, 40). Dexamethasone, a glucocorticoid class hormone, can also induce the KLF5 expression in MCF7 (our unpublished observation). It has been shown that the PR level is down-regulated in late pregnant stage and becomes undetectable during lactation (41). Thus the high levels of KLF5 protein and mRNA in the lactation stage are not likely due to Pg/PR. It is possible that Pg and PR only initiate the KLF5 expression induction during the early pregnancy stage. During the late pregnancy and lac-

tation stages, the KLF5 expression level could be maintained by other mechanism, such as glucocorticoid receptor. Further investigation will be required to clarify the mechanism by which the KLF5 expression in mammary glands is maintained during the lactation stage.

The Klf5 expression is up-regulated after pregnancy in mice (Fig. 1). It is well known that Pg starts to increase at early pregnancy so that the induction of Klf5 in mice is likely caused by Pg and PR. The PR knockout (PRKO) adult mice showed defects in normal breast development (34, 42). Because the klf5 homozygous KO mice are lethal, a breast-specific klf5 KO mouse model will be essential to evaluate the physiological role of Klf5 in normal breast development. We are currently characterizing the mouse mammary tumor virus-Cre; Klf5^{loxP/loxP} mice (43). Given the essential role of KLF5 in the Pg/PR signaling pathway, klf5 may be indispensable for normal breast development.

In conclusion, the expression of KLF5 is induced by Pg through PR in PR-positive breast cancer cell lines and probably in mouse mammary glands. Importantly, the induction of KLF5 partially mediates the pro-proliferation and dedifferentiation functions of Pg *in vitro*. KLF5 contributed to Pg-induced *CyclinA*, *Cdt1*, *E2F3*, and CK5 expression. Consistently, KLF5 and CK5 are coexpressed in ER-PR– basal type breast cancer cell lines. These findings suggest that the induction of KLF5 transcription factor by Pg contributes to Pg-induced breast cancer cell proliferation and dedifferentiation.

Materials and Methods

Tissue culture, transfection, and adenovirus infection

T47D, MCF7, BT474, BT-20, MD-MB-231, and other breast cancer cell lines were cultured as described previously (19, 20). All plasmids and siRNA were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The KLF5 siRNA has been described in our previous study (19). The target sequences of PR siRNA are 5'-TTTTCGACCCTCCAAGGACC-3' (no. 1) and 5'-TATGTAAGTTTCGAAAACC-3' (no. 2) (Ambion, Austin, TX). The KLF5 and control GFP adenoviruses have been described previously (18).

Reagents

Pg and MPA were purchased from Sigma (St. Louis, MO). The anti-KLF5 antibody has been described in our previous study (44). The anti-PR and anti-ER α antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-CK5 antibody was from Leica Microsystems (Bannockburn, IL). The anti-GAPDH antibody was from Cell Signaling Technology (Danvers, MA).

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Quantitative RT-PCR

Total RNA were isolated from tissues or cultured cells using TRIzol reagent (Invitrogen). Reverse transcriptions were performed using the Iscript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA). Quantitative PCR was performed on the ABI-7300 system using Absolute SYBR Green Fluorescein reagents (Thermo Fisher Scientific, Inc., Pittsburgh, PA). Primer sequences for *KLF5*, *CK5*, *SGK*, *CyclinA*, *Cdt1*, *E2F3*, and *GAPDH* will be provided upon request.

Dual luciferase assay

The 1.9-kb human *KLF5* gene promoter has been described previously (27). T47D cells were seeded in 24-well plates at 1.2×10^5 cells per well. The cells were transfected 24 h later with the *KLF5* promoter reporter plasmid, the *PR-B* expressing plasmid (a gift from Dr. Kathryn Horwitz, University of Colorado), and an internal control pRL-TK in triplicate. One day after transfection, the cells were serum starved for another day followed by 20 μ M MPA treatment. Luciferase activities were measured 24 h later by using the dual luciferase reporter assay system (Promega Corp., Madison, WI).

CHIP assay

T47D cells were incubated with 100 nM Pg or vehicle for 1 h. The ChIP assay was performed using the T47D cells following a protocol provided by Abcam (Cambridge, MA). The diluted DNA-protein complex was incubated with an equal amount of either anti-PR antibody or rabbit IgG antibody (Santa Cruz, CA) overnight at 4°C in the presence of protein A/G beads. Chromosomal DNA was purified and analyzed by PCR for the presence of the *KLF5* promoter. PCR primers used for amplifying the *KLF5* promoter (–647 to –447) were: 5′-TCCGCGTCTCCACCCTA-ATT-3′ and 5′-ATGAGCAGGGAGAGAGGCAG-3′. Two primers (5′-ACACCAGACCGCAGCTCCA-3′ and 5′-TCCATTGCT-GCTGTCTGATTGTAG-3′) were used to amplify the *KLF5* coding sequence (585–749). The *Bcl-2* promoter region (–629 to –388) with PR binding site (28) was used as a positive control. Two primers used were 5′-CTGGAGAGTGCTGAAGATTG-3′ and 5′-ACACTACAAGTAACACGGC-3′.

Thymidine incorporation assay

T47D cells were seeded in 24-well plates and were transfected with siRNA on the following day. One day after transfection, the cells were serum-starved for 1 d followed by 100 nM Pg treatment for another day. The cells were incubated in complete medium with 1 mCi/ml [³H]thymidine (MP Biomedical, Solon, OH) for 4 h. The incorporated [³H]thymidine was measured by the Beckman LS-6500 scintillation counter.

Statistical analysis

The luciferase assay and the DNA synthesis assay were conducted in triplicate. When appropriate, the data were pooled to generate means \pm SD and were analyzed by *t* test. *P* < 0.05 was considered to be significant.

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Address all correspondence and requests for reprints to: Ceshi Chen, Albany Medical College, Center for Cell biology and Cancer Research, Albany, New York. E-mail: chenceshi@hotmail.com or chenc@mail.amc.edu.

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The WWP1 Ubiquitin E3 Ligase Increases TRAIL Resistance in Breast Cancer

Zhongmei Zhou^{1,2}, Rong Liu², Ceshi Chen^{1,2*}

¹Key Laboratory of Animal Models and Human Disease Mechanisms of Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Kunming, Yunnan, 650223, China

²The Center for Cell Biology and Cancer Research, MS355, Albany Medical College, 47 New Scotland Ave. Albany, NY, 12208, USA

*Correspondence should be addressed to: chenc@mail.kiz.ac.cn

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ABSTRACT

WW domain containing E3 ubiquitin protein ligase 1 (WWP1) is an HECT domain-containing E3 ligase regulating apoptosis. The *WWP1* gene is frequently amplified and overexpressed in estrogen receptor α (ER α)-positive breast cancer. Inhibition of WWP1 by siRNA induced apoptosis in MCF7 and HCC1500. In this study, we demonstrate that WWP1 depletion by siRNA activated the extrinsic apoptotic pathway. WWP1 depletion-induced apoptosis was rescued by the overexpression of the wild type WWP1 but not the E3 ligase inactive WWP1-C890A mutant in MCF7 cells. In contrast, WWP1-C890A enhanced apoptosis, suggesting that the E3 ligase activity is required for WWP1 to promote cell survival. The expression levels of WWP1 in four breast cancer cell lines were specifically correlated with the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) resistance, but not TNF α and doxorubicin resistance. Both WWP1 depletion and dominant negative (DN) WWP1 overexpression increased the TRAIL-induced caspase-8 recruitment and apoptosis although WWP1 did not regulate FLIP and death receptor levels. Depletion of the initial caspase-8 blocked WWP1 inhibition-induced apoptosis in MCF7. These findings suggest that inhibition of WWP1 may be combined with TRAIL to suppress ER α -positive breast cancer cell survival.

INTRODUCTION

WWP1 is an HECT (homologous to the E6-associated protein carboxyl terminus) domain-containing ubiquitin E3 ligase. Accumulated evidence suggests that WWP1 plays an important role in cancers. We previously reported that the expression levels of WWP1 were frequently up-regulated in a subset of prostate and breast cancers^{1,2}. The expression of WWP1 in breast tumors correlated with the positive estrogen receptor (ER α) status^{3,4}. Forced overexpression of WWP1 promoted breast cell growth^{2,3,5}. WWP1 depletion in ER α -positive (MCF7, HCC1500, and T47D) breast cancer cell lines suppressed cell proliferation and induced apoptosis²⁻⁵. Similar results were observed in the MDCK canine kidney epithelial cell line⁶, PC-3 prostate cancer cell line¹, and HCT116 colon cancer cell line⁷. WWP1 depletion sensitized HCT116 cells to Doxorubicin and Cisplatin-induced apoptosis⁷.

WWP1 promotes cell proliferation and survival likely through multiple mechanisms. WWP1 regulates the EGF signaling pathway by modulating the ErbB4^{8,9}, EGFR, and ErbB2 levels⁵. Additionally, WWP1 negatively regulates the TGF- β signaling pathway by targeting TGF- β receptor 1 (T β R1)¹⁰, Smad2⁶, and Smad4¹¹ for degradation. WWP1 is the ubiquitin E3 ligase for apoptosis related transcription factors KLF5¹² and p63⁷.

The TNF-related apoptosis-inducing ligand (TRAIL) is a chemotherapeutic drug with strong anti-tumor activity in breast cancer and minimal cytotoxicity to most normal cells and tissues¹³. However, resistance to TRAIL-mediated apoptosis in breast cancer cells is a challenge for the successful application of TRAIL in cancer therapy¹⁴. TRAIL activates the

extrinsic apoptosis pathway through binding with death receptors (DR4 and DR5). Subsequently, an initial caspase, pro-caspase-8, is recruited for oligomerization and self-cleavage^{15, 16}. Activated caspase-8 induces apoptosis by directly cleaving effector caspases, e.g. caspase-3 and -7¹⁷. Additionally, caspase-8 can also indirectly activate caspase-9 through cleaving Bid¹⁸. The role of WWP1 in the TRAIL-induced extrinsic apoptosis pathway has not been reported. In this study, we demonstrate that WWP1 suppressed TRAIL-induced apoptosis through inhibiting caspase-8 activation in an E3 ligase activity dependent manner. Our findings suggest that WWP1 inhibition may be used to overcome TRAIL-resistance in ER α -positive breast cancers.

MATERIALS AND METHODS

Breast cancer cell lines

MCF7, HCC1500, MDA-MB-231, and SW527 breast cancer cell lines were cultured as described in our previous studies^{2, 5}.

Antibodies (Abs) and reagents

The mouse anti-human monoclonal WWP1 Ab is from Novus Biologicals (Littleton, CO). The anti-PARP, anti-caspase-8, -7, -9, and pJNK Abs are from Cell Signaling (Danvers, MA). The anti-FLIP Ab and human TRAIL are from Enzo Life Science (Plymouth Meeting, PA). The anti-FLAG M2 and anti- β -actin Abs are from Sigma. Western blotting (WB) was performed as described in our previous study⁴. The phycoerythrin (PE) conjugated anti-DR4, DR5, and control mouse IgG are from R&D systems, Inc (Minneapolis, MN). The membrane protein levels of DR4 and DR5 were analyzed by flow cytometry as described in our previous study⁵.

Knockdown (KD) of WWP1 and caspase-8

WWP1 KD by siRNA was performed as described in our previous studies^{2, 12}. All siRNA transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). All chemically synthesized siRNAs were purchased from Ambion (Austin, TX) and transfected at 10-25 nM final concentration. The siRNA target sequence for the *CASP8* gene is 5'- GATCAGAATTGAGGTCTTT- 3'.

A lentiviral pSIH-H1-puro vector was used to express WWP1 shRNAs. Two WWP1 shRNAs were designed according to the WWP1 siRNA target sequences (5'-GAGTTGATGATCGTAGAAG -3' and 5'- GACCAAAGCTTTCCTTGAT -3'). The luciferase (Luc) shRNA was used as the negative control. All lentiviral plasmids and the packing plasmids were co-transfected into HEK293FT cells. Lentiviruses were collected at 72 hrs after transfection and used to transduce MCF7 cells in a 24-well plate. Twenty-four hours after transduction, puromycin (2 µg/ml) was added to select drug resistant cell populations.

Stable expression of WWP1 in MCF7

The WWP1 siRNA resistant pLenti6-WWP1 vector has been described in our previous study⁵. The WWP1 siRNA resistant pLenti6-WWP1-C890A vector was generated using the same strategy. The pLenti6-lacZ vector was used as the negative control. All plasmids were transfected into HEK293FT packing cells. Lentiviruses were collected at 72 hrs after transfection and used to transduce MCF7 cells in a 6-well plate. Forty eight hrs after transduction, the cells were trypsinized and seeded into 60-mm dishes. Blasticidin (10 µg/ml) was added to select drug-resistant cells.

Measurement of cell viability and apoptosis

Breast cancer cells were transfected with the WWP1 siRNA and Luc control siRNA for two days. If applied, different concentrations of TRAIL, TNF α , and Doxorubicin were added for 24 hrs. The propidium iodide (PI) staining, flow cytometry, cell viability assays were performed as described in our previous study². The student t-test was used to

determine statistical differences between the experimental and control groups. The result was considered statistically significant if a *P*-value is smaller than 0.05. MCF7 is a caspase-3 deficient cell line¹⁹. Cleaved PARP and caspase-8, -9, and -7 protein levels were detected by WB.

Immunoprecipitation of the death-inducing signaling complex (DISC)

TRAIL-induced DISC was immunoprecipitated and subjected to immunoblotting analysis. In brief, 1×10^7 MCF7 (LacZ, WWP1, WWP1m, Lucsh, and WWP1sh) cells were digested and treated with 1 μ g Flag-TRAIL (Alexis, San Diego, CA) and 4.6 μ g anti-Flag monoclonal M2 Ab for 30 mins at 37°C. Flag-TRAIL-induced DISC was immunoprecipitated with protein A/G Plus-Agrose (Santa Cruz Biotechnology, Santa Cruz, CA). For the unstimulated control, the cells were first lysed then incubated with mixed Flag-TRAIL/anti-Flag M2 Ab/protein A/G Plus-Agrose.

RESULTS

WWP1 KD activated the extrinsic apoptotic pathway

Previously, we reported that WWP1 KD by siRNA induced apoptosis in MCF7 and HCC1500 breast cancer cells². Caspase-9, -7, and/or -3 were activated in both cell lines². To determine whether the WWP1 KD activates the extrinsic apoptotic pathway, we transfected MCF7 and HCC1500 cells with the WWP1 siRNA and Luc negative control siRNA, respectively and tested the activation of the initial caspase-8 and c-Jun NH2-terminal kinase (JNK), both contribute to the TRAIL-activated extrinsic apoptotic pathway^{20, 21}. After WWP1 was knocked down, the cleaved caspase-8 and pJNK protein levels were dramatically upregulated in both MCF7 and HCC1500 cells (Fig. 1A). These findings suggest that WWP1 depletion activates the extrinsic apoptotic pathway.

WWP1 siRNA-induced apoptosis can be rescued by the wild type (WT) WWP1 but not the catalytic inactive WWP1-C890 mutant

To eliminate the possible off-target effects of siRNA and to determine if the WWP1 E3 ligase activity is required for WWP1 to promote breast cancer cell survival, we performed a rescue experiment in MCF7. The siRNA-resistant WT WWP1 and the catalytic inactive WWP1-C890 mutant were overexpressed in MCF7 respectively. The WWP1 siRNA-induced caspase-8, -9, -7, and PARP cleavage and JNK activation were almost completely blocked by WT WWP1, but not by the catalytic inactive WWP1-C890 mutant (Fig. 1B). In contrast, WWP1-C890 increased the WWP1 siRNA-induced caspase-8, -7, and PARP cleavage and JNK activation, suggesting that WWP1-C890 functions as a dominant negative (DN) form of WWP1.

High expression levels of WWP1 correlated with the TRAIL-resistance in four breast cancer cell lines

Since WWP1 inhibition activates the extrinsic apoptotic pathway, we wondered if WWP1 suppresses TRAIL- and TNF α -induced apoptosis in breast cancer. WWP1 is highly expressed in ER α -positive breast cancer cell lines including MCF7 and HCC1500, but not in ER α -negative breast cancer cell lines including MDA-MB-231 and SW527 (Fig. 2A). Interestingly, MDA-MB-231 and SW527 cells were sensitive to TRAIL while MCF7 and HCC1500 cells were resistant to TRAIL (Fig. 2B). However, their responses to TNF α were opposite (Fig. 2C). These breast cancer cell lines showed similar sensitivities to the DNA damage drug Doxorubicin (Fig. 2D).

The inhibition of WWP1 sensitized TRAIL-resistant breast cancer cells to TRAIL-induced apoptosis

Since the inhibition of WWP1 by siRNA or DN WWP1 activated the extrinsic apoptotic pathway and the expression levels of WWP1 correlated with TRAIL-resistance in four breast cancer cell lines, we then tested if the inhibition of WWP1 sensitizes TRAIL-resistant breast cancer cells to TRAIL-induced apoptosis. As shown in Fig. 3A, WWP1 depletion by siRNA in combination with TRAIL (25 ng/ml) induced more cleavage of caspase-8, -7 and PARP than WWP1 depletion or TRAIL alone did in both MCF7 and HCC1500 cells. The increases of apoptosis in MCF7 and HCC1500 were further confirmed by the PI staining analysis (Fig. 3A). Similarly, the inhibition of WWP1 by DN WWP1 sensitized MCF7 cells to TRAIL-induced caspase-8, -7, and PARP cleavage (Fig. 3B). In contrast, the overexpression of WT WWP1 in MCF7 almost

completely blocked TRAIL-induced caspase-8, -7, and PARP cleavage (Fig. 3B). Stable WWP1 KD in MCF7 by two different shRNAs also increased TRAIL-induced caspase-8 and -7 activation (Fig. 3C) as well as the cell viability decrease (Fig. 3D). These results suggest that the inhibition of WWP1 by either siRNA or DN WWP1 sensitizes TRAIL-resistant breast cancer cells to TRAIL-induced apoptosis.

The inhibition of WWP1 did not regulate the FLIP and death receptor (DR) protein levels but increased the caspase-8 recruitment into the DISC

To explore the possible mechanism by which WWP1 promoted TRAIL-resistance in breast cancer, we first examined the FLIP_{L/S} (long/short isoforms) protein levels in MCF7 because the WWP1 closely related family member Itch/AIP4 has been shown to target FLIP for proteasomal degradation²²⁻²⁴. When WWP1 was stably knocked down by two different shRNA in MCF7, the FLIP_L and FLIP_S protein levels were unchanged (Fig. 4A). Consistently, the overexpression of WT or DN WWP1 did not alter the FLIP protein levels (Fig. 4A). WWP1 KD did not change the protein levels of FADD, RIP1, ASK1, and MEKK2 in MCF7 and HCC1500 cells (data not shown). The protein levels of DR4 and DR5 were examined by flow cytometry. As shown in Fig. 4B, MCF7 cells only express DR5. The expression of DR4 in MCF7 was undetectable. The DR5 levels were not changed by WWP1 KD in MCF7 (Fig. 4B).

Flag-TRAIL-induced DISC was immunoprecipitated from MCF7 cells overexpressing LacZ, WWP1, DN-WWP1, Lucsh, and WWP1sh. As shown in Fig. 4C, FADD and caspase-8 were not co-immunoprecipitated with Flag-TRAIL and DR5 in unstimulated

control cells. Interestingly, the endogenous WWP1 protein was co-immunoprecipitated with Flag-TRAIL and DR5. Importantly, both DN WWP1 and WWP1 KD increased the recruitment of caspase-8 while WT WWP1 had little effect. DN WWP1 and WWP1 KD did not increase the recruitment of FADD (Fig. 4C).

Inhibition of WWP1-induced apoptosis depended on caspase-8

To better understand the signaling pathway by which WWP1 promoted breast cancer cell survival and TRAIL-resistance, we tested the role of caspase-8 by knocking down caspase-8 in MCF7 cells. As shown in Fig. 5A, KD of caspase-8 protected MCF7 cells from the WWP1 siRNA-induced apoptosis, as measured by the cleavage of caspase-8 -7 and PARP. The results were confirmed by the cell viability assay. Indeed, KD of caspase-8 significantly rescued the WWP1 siRNA-induced loss of cell viability (Fig. 5B). Similarly, DN WWP1-induced apoptosis was also blocked by the depletion of caspase-8 in MCF7 in the absence and presence of TRAIL (Fig. 5C-D). Interestingly, caspase-8 depletion also partially rescued loss of MCF7 cell viability induced by the long term TRAIL treatment in combination with Lucsi transfection (Fig. 5D). These results clearly indicate that caspase-8 is essential for WWP1 to promote cancer cell survival and TRAIL resistance.

DISCUSSION

In this study, we present several lines of evidence to suggest that WWP1 may be associated with TRAIL-resistance in a subset of breast cancers for the first time. Firstly, the expression of WWP1 positively correlated with the TRAIL resistance in four breast cancer cell lines. Secondly, the inhibition of WWP1 increased the TRAIL-induced apoptosis in TRAIL-resistant MCF7 and HCC1500. Additionally, the overexpression of WWP1 decreased the TRAIL-induced apoptosis in an E3 ligase activity dependent manner. Lastly, the inhibition of WWP1 induced apoptosis through caspase-8, an initial caspase in the extrinsic apoptotic pathway that is also essential for TRAIL to induce cell death.

Our data suggest that WWP1 may be a biomarker and regulator of TRAIL sensitivity. Recently, Rahman M et al., reported that ER/PR/HER2 triple-negative breast cancer cells with a mesenchymal phenotype are more sensitive to TRAIL than ER α -positive breast cancer cells¹⁴. The WWP1 ubiquitin E3 ligase is frequently overexpressed in ER α -positive breast cancer cell lines and primary tumors²⁻⁴. Interestingly, the WWP1^{high} breast cancer cell lines, such as MCF7, HCC1500, and T47D are resistant to TRAIL while the WWP1^{low} breast cancer cell lines, such as MDA-MB-231, SW527, Hs578T, MDA-MB-157, and ZR-75-1 are sensitive to TRAIL (This study and references^{2, 14, 25, 26}). KD of WWP1 directly induced apoptosis and increased the TRAIL efficacy in TRAIL-resistant breast cancer cell lines. Therefore, WWP1 targeted therapy in combination with TRAIL may be an attractive strategy to treat ER α -positive breast cancer.

Although WWP1 functioned through the caspase-8 dependent extrinsic apoptotic pathway and WWP1's E3 ligase activity, the exact molecular mechanism by which WWP1 specifically associates with TRAIL resistance, but not with TNF α resistance, in breast cancer cells is still unclear at present. FLIP has been well documented to cause TRAIL resistance in cancers²⁷⁻²⁹. The WWP1 family member Itch promotes FLIP degradation²²⁻²⁴; however, WWP1 appears not to regulate the FLIP levels in MCF7. Similarly, WWP1 does not regulate the DR4/5, FADD, RIP, and caspase-8 protein levels as well. Similar to Itch, WWP1 was recruited into DISC after the TRAIL treatment. The increase of the pro-caspase-8 recruitment may explain why inhibition of WWP1 sensitized MCF7 cancer cells to TRAIL. Overexpression of WT WWP1 did not decrease the pro-caspase-8 recruitment in MCF7, indicating other mechanisms may be involved. However, WWP1 did not ubiquitinate caspase-8 (data not shown). The WWP1 known substrates, including KLF5¹², p63⁷, and ErbB4^{8,9}, have not been shown to regulate TRAIL-induced apoptosis. Thus, the direct mechanism of action of WWP1 in the TRAIL-induced apoptosis needs further elucidated in the future.

In summary, we found that the overexpression of WWP1 correlated with the TRAIL resistance in several human breast cancer cell lines. Functionally, the inhibition of WWP1 by RNAi or the overexpression of DN WWP1 induced apoptosis and sensitized breast cancer cells to TRAIL-induced apoptosis. The WWP1 pro-survival function depended on its E3 ligase activity and caspase-8. These results indicate that WWP1 could be a potential biomarker and therapeutic target in ER α -positive TRAIL-resistant breast cancers.

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FIGURE LEGENDS

Figure 1. The inhibition of WWP1 activated the extrinsic apoptotic pathway

A. WWP1 KD by siRNA increased the pJNK and cleaved caspase-8 levels in both MCF7 and HCC1500 cells. Lucsi was used as the negative control for the WWP1 siRNA². The cells were transfected with siRNAs for two days. Apoptosis is indicated by the cleavage of caspase-8, -9, -7 and PARP. The protein levels were detected by WB. The molecular weight for each protein is indicated at the right side of panels. β -actin was used as the loading control. CL means cleaved.

B. The overexpression of WT WWP1*, but not the ligase inactive WWP1-C890A (WWP1m*), rescued the WWP1 (W) siRNA-induced apoptosis in MCF7. Luc (L) siRNA was used as the negative control. Both WT WWP1* and WWP1m* were silently mutated to resist to the WWP1 siRNA. WWP1m* enhanced WWP1 siRNA-induced apoptosis in MCF7. LacZ was used as the negative control for WWP1.

Figure 2. High expression levels of WWP1 were specifically associated with the TRAIL resistance in four breast cancer cell lines

A. Expression levels of the WWP1 protein in four breast cell lines were detected by WB. β -actin was used as the loading control. The quantitative ratios (WWP1/ β -actin) by using the Multi Gauge software are indicated below the panel.

B. The TRAIL sensitivity of four breast cancer cell lines was measured by the Sulphorhodamine-B (SRB) assay. MCF7 and HCC1500 were resistant to TRAIL; but MDA-MB-231 and SW527 were sensitive to TRAIL. Different concentration of TRAIL (0-50 ng/ml) were used to treat cancer cells for one day.

C. The TNF α (0-10 ng/ml) sensitivity of four breast cancer cell lines

D. The Doxorubicin (0-500 nM) sensitivity of four breast cancer cell lines

Figure 3. The inhibition of WWP1 increased the TRAIL sensitivity in

TRAIL-resistant breast cancer cell lines

A. WWP1 KD by siRNA and TRAIL (25 ng/ml) additively induced apoptosis in MCF7 and HCC1500 as detected by WB and flow cytometry. The cells were transfected with siRNAs for two days and were treated with TRAIL for one day. Apoptosis is indicated by the cleavage of caspase-8, -7 and PARP in the left panel. The right panel shows the quantitative apoptotic data (sub-G1 cells), as detected by the PI staining and flow cytometry, *, $P < 0.05$; **, $P < 0.01$ (t-test).

B. The overexpression of WT WWP1* reduced, but WWP1m* increased, the TRAIL (25 ng/ml) -induced apoptosis in MCF7. Apoptosis is indicated by the cleavage of caspase-8, -7 and PARP.

C. WWP1 KD by two different shRNA and TRAIL (25 ng/ml) additively induced apoptosis in MCF7 as detected by WB. Apoptosis is indicated by the cleavage of caspase-8 and -7. The 41/43 KDa cleaved caspase-8 bands are shown (*).

D. WWP1 KD by two different shRNAs and TRAIL additively decreased the cell viability in MCF7 as detected by the SRB assay. The cells were treated with 25 ng/ml TRAIL for two days. The killing effects of TRAIL are shown.

Figure 4. The inhibition of WWP1 did not regulate the FLIP and DR protein levels but increased the caspase-8 recruitment into DISC in MCF7

- A.** WWP1 KD by two different shRNA did not alter the FLIP_L (long isoform) and FLIP_S (short isoform) protein levels. The overexpression of WT WWP1* or WWP1m* did not change the FLIP protein levels as well.
- B.** WWP1 KD by two different shRNA did not alter the DR4 and DR5 protein levels, as determined by flow cytometry. IgG was used as the negative control. No DR4 was detected in MCF7 cells by flow cytometry.
- C.** DN-WWP1 and WWP1 KD increased the caspase-8 recruitment into DISC in MCF7. MCF7 cells overexpressing LacZ, WWP1, DN-WWP1, Lucsh, and WWP1sh#1 were immunoprecipitated by Flag-TRAIL/anti-Flag M2 Ab before (+) and after (-) the cells were lysed and subjected to immunoblotting with different Abs, as indicated.

Figure 5. The inhibition of WWP1 induced apoptosis partially through caspase 8

- A.** Caspase-8 KD by siRNA reduced the WWP1 siRNA-induced apoptosis in MCF7. The cells were transfected with siRNAs for two days. Apoptosis is indicated by the cleavage of caspase-7 and PARP.
- B.** Caspase-8 KD by siRNA significantly rescued the WWP1 siRNA-induced apoptosis in MCF7. Cell viability was measured by the SRB assay, *, $P < 0.05$ (t-test).
- C.** WWP1m* increased the TRAIL-induced apoptosis partially through Caspase-8. Caspase-8 KD by siRNA dramatically blocked the WWP1m* induced apoptosis in MCF7 in the absence and presence of TRAIL, as determined by WB. The cells were transfected with siRNAs for one day and were treated with TRAIL for one day. Apoptosis is indicated by the cleavage of caspase-7 and PARP.

D. Caspase-8 KD significantly rescued the WWP1m* induced cell viability decrease in MCF7 in the absence and presence of TRAIL, as determined by the SRB assay, **, $P < 0.01$ (t-test). The cells were transfected with siRNAs for three days and were treated with TRAIL for two days.

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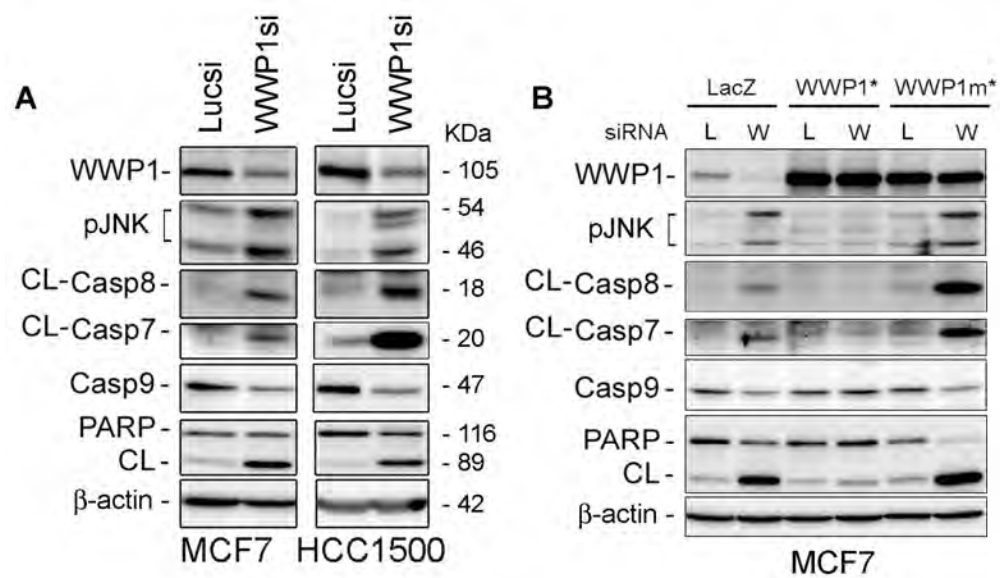


Fig. 1

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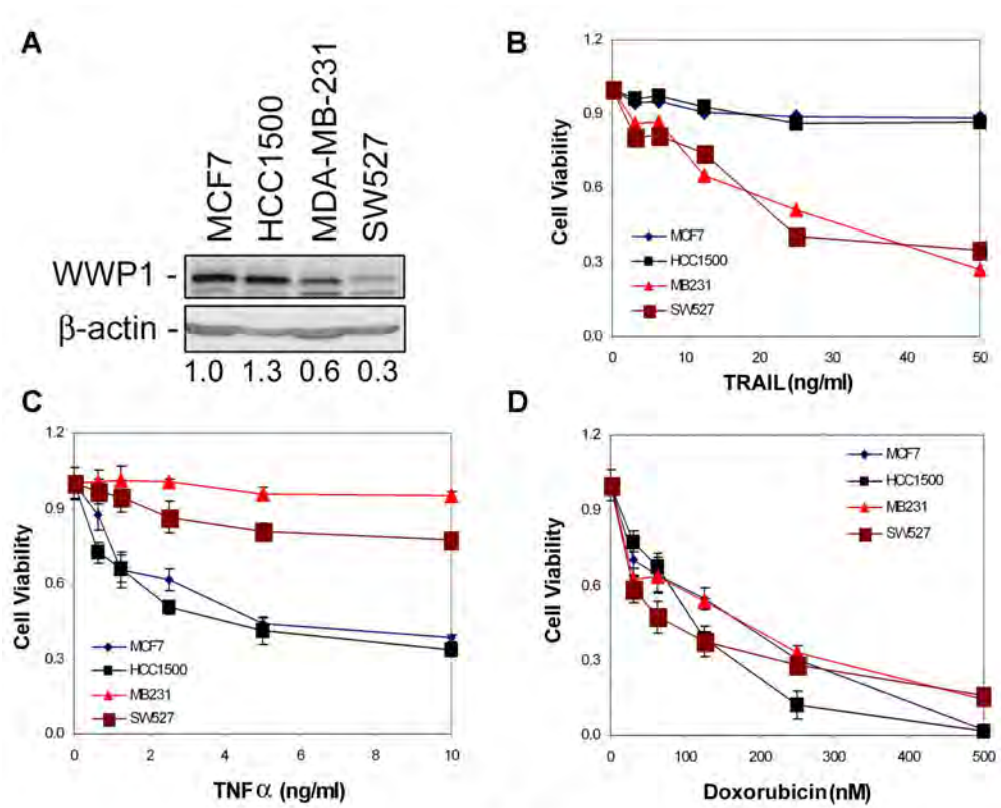


Fig. 2

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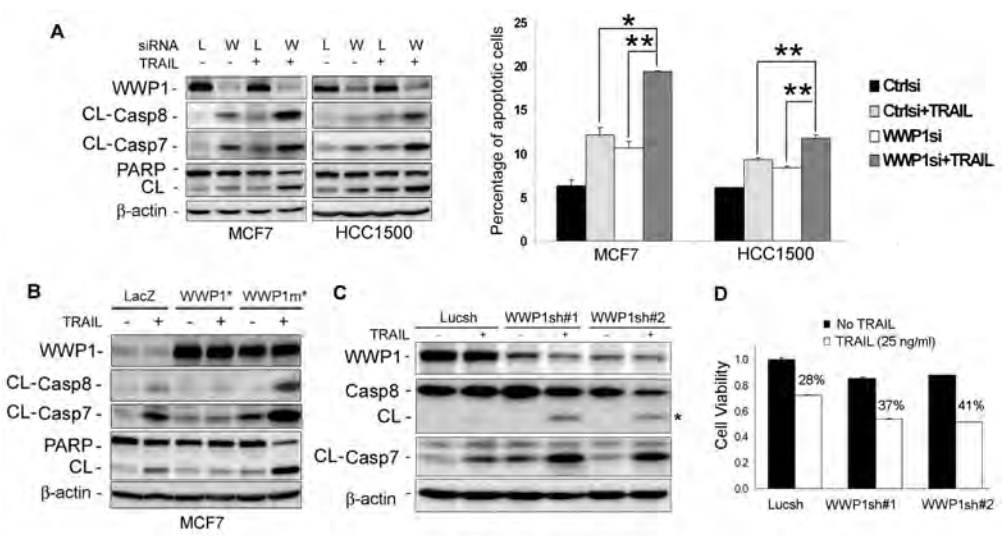


Fig. 3

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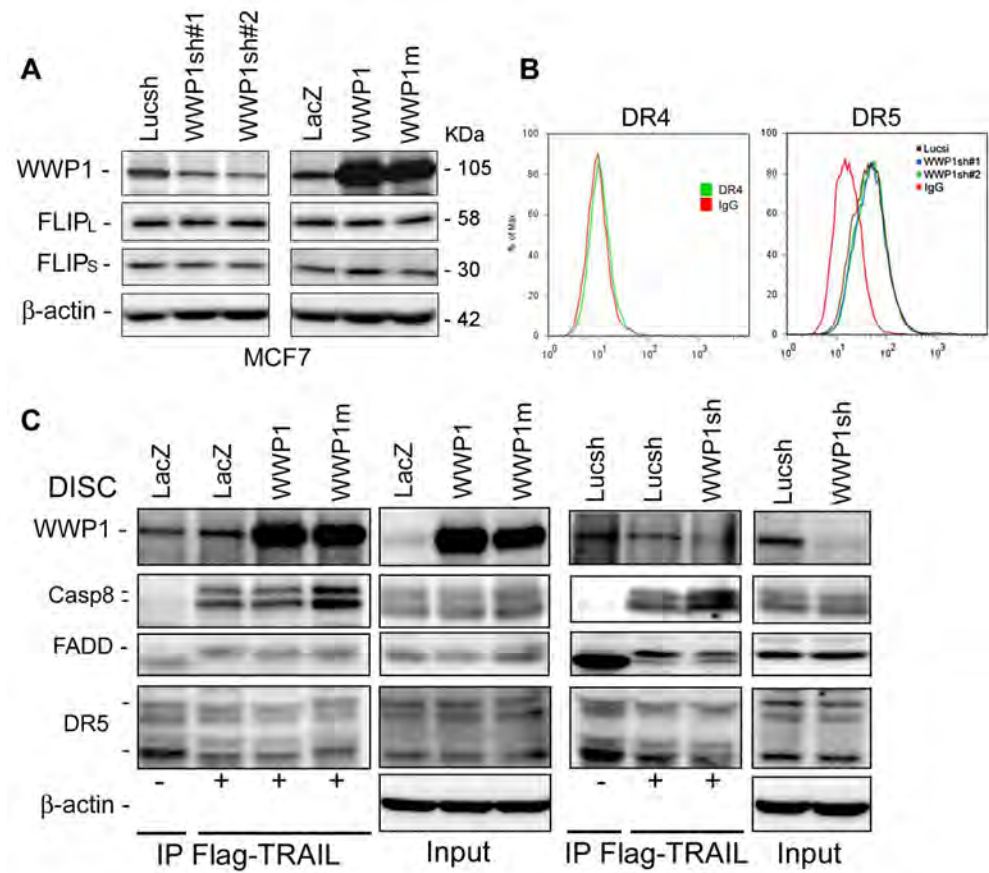


Fig.4

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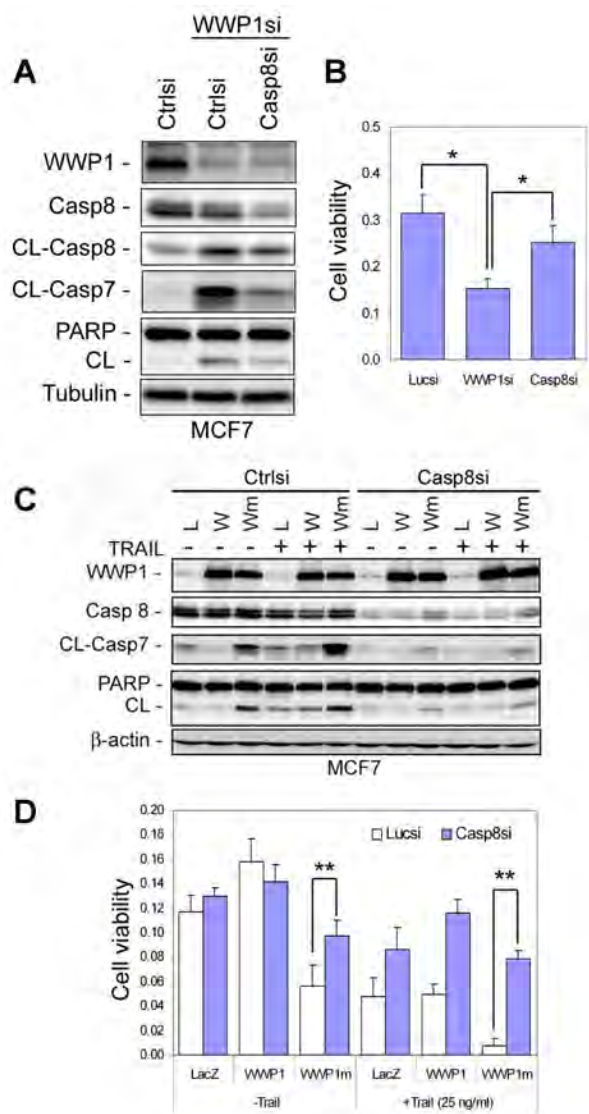


Fig. 5

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WWP1: a versatile ubiquitin E3 ligase in signaling and diseases

Xu Zhi¹, Ceshi Chen^{1,2}

¹The Center for Cell Biology and Cancer Research, Albany Medical College, 47, New Scotland Ave. Albany, NY, USA, 12208,

²Key Laboratory of Animal Models and Human Disease Mechanisms of Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Kunming, Yunnan, China, 650223

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1. ABSTRACT

WW domain-containing E3 ubiquitin protein ligase 1 (WWP1) is a multifunction protein containing an N-terminal C2 domain, four tandem WW domains for substrate binding, and a C-terminal catalytic HECT domain for ubiquitin transferring. WWP1 has been suggested to function as the E3 ligase for several PY motif containing proteins, such as Smad2, KLF5, p63, ErbB4/HER4, RUNX2, RNF11, SPG20, and Gag, as well as several non-PY motif containing proteins, such as TbetaR1, Smad4, KLF2, and EPS15. WWP1 regulates a variety of cellular biological processes including protein trafficking and degradation, signaling, transcription, and viral budding. WWP1 has been implicated in several diseases, such as cancers, infectious diseases, neurological diseases, and aging. In this review article, we extensively summarize the current knowledge of WWP1 with special emphasis on the roles and action of mechanism of WWP1 in signaling and human diseases.

2. INTRODUCTION

WWP1 (WW domain-containing E3 ubiquitin protein ligase 1), TIUL1 (TGIF-interacting ubiquitin ligase 1) (1), or AIP5 (Atropin-1-interacting protein 5) (2) is a C2-WW-HECT (homologous to E6-AP COOH terminus) type ubiquitin E3 ligase. The C2-WW-HECT E3 ligase family contains nine members, i.e. NEDD4-1 NEDD4-2/NEDD4L, WWP1, WWP2/AIP2, Itch/AIP4, SMURF1, SMURF2, NEDL1, and NEDL2. All of them share three common functional domains: an N-terminal Ca²⁺/lipid-binding C2 domain, 2-4 substrate-binding WW domains, and a C-terminal catalytic HECT domain (3). The NEDD4-like family is involved in a diverse variety of cellular processes, such as membrane protein trafficking, protein degradation, signaling, transcription, and apoptosis (3-6). Several family members have been implicated in cancers, hypertension, neurological diseases, bone metabolism, and immune diseases (3). Accumulating evidence indicates that WWP1 plays important roles in cancers, infectious diseases,

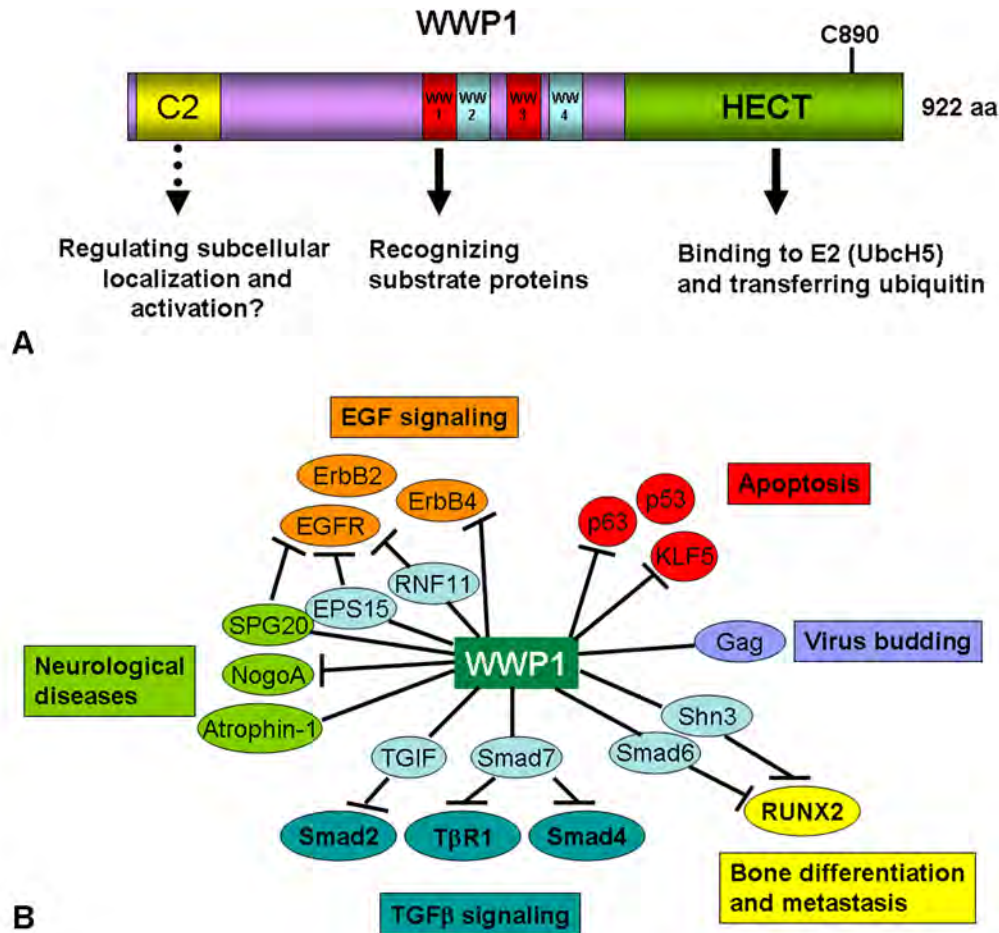


Figure 1. A. The diagram of the WWP1 protein domains. The WWP1 protein contains 922 amino acid residues. The C2 domain at N-terminus is responsible for membrane and protein binding. The four WW domains in the central region are responsible for the interaction with substrate proteins. The WW1 and WW3 are type I WW domains that recognize PY motifs. The HECT domain at the C-terminus is responsible for the ubiquitin-protein ligase activity. The Cysteine-890 is the catalytic activation site. B. WWP1 regulates different substrates in different cellular processes.

and neurological diseases. We attempt to comprehensively summarize the recent progresses of WWP1 investigations including the protein structure and functions, gene expression, regulation, substrates, and animal models.

3. BIOCHEMISTRY OF WWP1

3.1. WWP1 protein structure

The human WWP1 protein contains 922 amino acid residues with the molecular weight of ~110 KDa. It contains a C2 domain, four WW domains, and a HECT domain (Figure 1a). The N-terminal C2 domain may mediate the protein-protein interaction, Ca^{2+} dependent membrane binding, and enzyme activation according to the studies on other WWP1 family members, such as NEDD4 (7-9) and SMURF2 (10). Consistently, WWP1 is predominately localized to the endosome (11).

WWP1 has four tandem WW domains in its central part. The WW domain is composed of 38-40 semi-conserved residues with two conserved tryptophans (W)

that are spaced 20-22 residues apart and has been shown to mediate protein-protein interaction (12). The WW domain binds to Pro-rich polypeptide ligands, such as PPXY (PY motif), PPLP, PPR, or p(S/T)P (13). WW#1 and #3 of WWP1 are type I WW domains that bind to PY motifs with higher affinities as compared to WW#2 and #4 (14-16). The C-terminus of WWP1 possesses a HECT domain that interacts with an ubiquitin (Ub) conjugation enzyme (i.e. UbcH5) and is responsible for its ubiquitin E3 ligase enzyme activity (17). E3 ligases are key enzymes for protein ubiquitination because they determine substrate specificity. To date, 28 out of over 600 ubiquitin E3 ligases have been identified to be HECT type E3s in mammalian cells. The catalytic cysteine (C) 890 of human WWP1 is responsible for ubiquitin transferring (1).

3.2. WWP1 gene expression

The WWP1 gene is highly conserved among different animals including *C. elegans*, *chicken*, *mouse*, and *human*. The *C. elegans wwp-1* gene encodes 792 amino acid residues (18). The human WWP1 gene is localized on

chromosome 8q21 and encompasses 26 exons, which span at least 142 kb DNA (19). The 8q21 chromosome region is frequently amplified in several human cancers, including prostate and breast cancers. We reported that gain of gene copy number for *WWP1* was detected in 31-51% of prostate and breast cancers (20, 21). Furthermore, the *WWP1* gene is mutated in human cancers. In 30 prostate cancer samples, two sequence alterations that change the *WWP1* protein sequence were detected (20). However, the functions of these mutations have not been characterized.

The *WWP1* mRNA is ubiquitously expressed in multiple tissues. Mosser *et al.*, investigated the *WWP1* mRNA expression by Northern blot and found that *WWP1* is highly expressed in the heart and skeletal muscle (14). This result was confirmed by an independent Northern blot study (2). Quantitative PCR results indicated that *WWP1* is highly expressed in the liver, heart, and testis (22). Several studies showed that the *WWP1* mRNA is normally expressed in the prostate and breast at low levels (22, 23); however, it is dramatically elevated in a subset of prostate and breast tumors (20, 21, 24).

The *WWP1* mRNA undergoes alternative splicing. *WWP1* shows two splice variants in different human tissues by Northern blot (2, 14). Flasz *et al.*, identified six *WWP1* splice variants by RT-PCR in the T47D human breast cancer cell line (25). By Northern blot, Huang *et al.*, demonstrated that mouse *Wwp1* mRNA has four different splice variants that are highly expressed in the heart, kidney, liver, and testis (18). The relative ratios of different splice products appear to be tissue-specific. The *WWP1* mRNA alternative splicing may be functional relevant. Some isoforms without the C2 domain may have distinct or dominant negative functions (25). We found an extra, smaller *WWP1* protein band below the wild type *WWP1* band in two prostate cancer cell lines PC-3 and LAPC-4 (20).

3.3. WWP1 protein subcellular localization: membrane, cytoplasm, or nucleus?

The *WWP1* protein is predominantly localized in the early endosome, in the cytoplasm, and occasionally in the nucleus. By immunofluorescence (IF) analysis, Seo *et al.*, demonstrated that exogenous *WWP1*/Tiul1 protein is expressed in both the nucleus and cytoplasm of MDCK canine kidney epithelial cells (1). Consistently, we found that *WWP1* is expressed in both the nucleus and cytoplasm of the 22Rv1 prostate cancer cell line (11, 23). It is worth pointing out that the *WWP1* protein is predominantly localized in cytoplasmic punctate membrane structures and occasionally localized in the nucleus (11). Flasz *et al.*, demonstrated that *WWP1* is localized to the early endosome in C2C12 murine skeletal muscle-derived myoblast cells (26). By immunohistochemistry (IHC), Huu *et al.*, detected both nuclear and cytoplasmic endogenous *WWP1* protein expression in breast tumors (24). However, we only detected the cytoplasmic *WWP1* protein expression in breast tumors using a mouse monoclonal anti-*WWP1* antibody (27). Thus, all studies support the cytoplasmic localization of *WWP1* while the nuclear localization of *WWP1* is context dependent.

3.4. Regulation of WWP1

WWP1 has been shown to be regulated at the transcriptional level. *WWP1* mRNA levels are increased during osteoblast differentiation (28). We found that transforming growth factor β (TGF β) induced *WWP1* mRNA expression in HaCaT and PC3 cells (3). *WWP1* has been suggested to suppress TGF β signaling through targeting Smad2 and a type I receptor (T β R1) for degradation (1, 22). It is possible that the induction of *WWP1* by TGF β is a negative feedback mechanism for controlling the TGF β signaling pathway. Additionally, we found that DNA damage chemotherapeutic drugs (Doxorubicin and Cisplatin) induced *WWP1* in a p53-dependent manner in MCF10A and HCT116 cells (15). However, *Wwp1* mRNA levels decreased after exposure to UV- or γ -irradiation in p53 wild type mouse embryo fibroblast cells (29). Thus, DNA damage may regulate the *WWP1* expression through p53 in a cell line dependent manner. Nevertheless, these results imply that *WWP1* is a downstream target gene of p53. Interestingly, *WWP1* negatively regulates the activities of p53 and its closely related family member p63 (15, 29). Further investigations are necessary to elucidate the mechanism by which *WWP1* is regulated by p53.

In addition, *WWP1* is frequently up-regulated in reproductive hormone related cancers including prostate and breast cancers (20, 24, 27). *WWP1* has been suggested to be induced by androgen in the LAPC9 prostate cancer cell line (30). However, this result could not be verified in the LNCaP prostate cancer cell line (unpublished observation). We reported that the *WWP1* protein expression correlates with the estrogen receptor (ER α) positive status in breast tumors (27). However, *WWP1* is not an estrogen responsive gene in the ER α -positive MCF7 breast cancer cell line (unpublished observation). Whether the *WWP1* expression is regulated by other hormones is unclear.

We previously showed that the *WWP1* protein is degraded by the 26S proteasome (3). The half-life of *WWP1* protein is about 3 hours in 22Rv1 cells. The proteasome inhibitor MG132 could dramatically increase the *WWP1* protein levels in MCF10A and 22RV1 cells (3).

Finally, the cellular localization of *WWP1* is regulated by the Notch signaling. Notch could deplete *WWP1* out of the nucleus, and co-localize with *WWP1* in early endosomes. In non-differentiated C2C12 cells, *WWP1* is distributed in both the nucleus and cytoplasm. When the cells were induced to differentiate, the localization of *WWP1* was exclusively in the cytoplasm (26). Whether *WWP1* is regulated by other posttranslational modifications, such as phosphorylation, is currently unknown. Understanding the regulatory mechanisms of *WWP1* will help us design effective strategies for targeting the *WWP1* pathway in diseases.

4. WWP1-MODIFIED SUBSTRATE PROTEINS

As an ubiquitin E3 ligase, *WWP1* interacts with a variety of substrate proteins and regulates their expression

Table 1. The list of WWP1 interacting proteins

WWP1 interacting proteins	Regulation	Functions	References
PY motif containing substrates			
ErbB4/HER4	Degradation	Differentiation and cancer	(16, 38)
p63	Degradation	Apoptosis and cancer	(15)
KLF5	Degradation	Proliferation, apoptosis, and cancer	(23, 49)
RNF11	Ubiquitination, no degradation	EGFR and ErbB2 degradation	(11)
Spartin (SPG20)	mUb, no degradation	Troyer syndrome	(44, 45)
Nogo-A	Degradation	Central nervous system regeneration	(66)
Smad2	Degradation	TGF β signaling	(1, 20)
Smad6	No degradation	Adaptor for RUNX2	(28)
Smad7	No degradation	TGF β signaling	(1, 22)
RUNX2	Degradation	Osteoblast differentiation	(28, 55, 56)
Gag	mUb, no degradation	Virus budding	(59, 60, 63, 70)
Atrophin-1		Muscular dystrophy	(2)
WBP-1/2		Co-activator of PR and ER	(70, 71)
RasGAP		Apoptosis and migration	(70)
p53-BP2			(70)
IL6R α		Inflammatory response	(70)
NF-E2 (p45)		Globin gene transcription	(14)
Non-PY motif containing substrates			
T β R1	Degradation	TGF β signaling	(1, 22)
Smad4	Degradation	TGF β signaling	(20, 31)
TGIF	No degradation	Adaptor for Smad2	(1)
Shn3	No degradation	Adaptor for RUNX2	(55)
p53	Subcellular localization	Apoptosis	(29)
KLF2	Degradation	Transcription	(50)
EPS15	mUb, no degradation	Trafficking	(3)

Abbreviations: mUb, mono-ubiquitination

levels and activities. To date, WWP1 has been shown to function as the E3 ligase for T β R1, Smad2, ErbB4/HER4, RNF11, SPG20, KLF5, p63, RUNX2, and others (Figure 1b and Table 1).

4.1. TGF β signaling pathway

The TGF β superfamily regulates cell proliferation, differentiation, apoptosis, and migration in numerous cell types. TGF β ligands bind to a type II receptor (T β R2), which recruits and phosphorylates T β R1. Then, T β R1 recruits and phosphorylates receptor-regulated Smads (R-Smads), Smad2 and Smad3. The phosphorylated R-Smads bind to the common Smad4, enter the nucleus, and form transcription complexes to regulate gene expression.

Accumulating evidence suggests that WWP1 negatively regulates the TGF β signaling pathway (1, 20, 22). WWP1 inhibits TGF β -induced transcriptional activities as well as PAI-1 and JunB expression (1, 22). In addition, overexpression of WWP1 in MDCK cells reduces the TGF β -induced growth inhibition (1). Among eight Smads, WWP1 can strongly interact with Smad2, 3, 6, 7 proteins, weakly with Smad1 and 5, but not with Smad4 and Smad8, which do not contain a PY motif (1, 22). WWP1 directly targets Smad2 for ubiquitination and degradation in the presence of TGIF (1, 22). Two independent studies suggest that WWP1 targets T β R1 for ubiquitin-mediated degradation through the Smad7 adaptor (1, 22). Morén *et al.*, reported that WWP1 uses Smad7 to induce Smad4 ubiquitination and degradation (31). Thus, WWP1 inhibits the TGF- β signaling pathway directly or indirectly by targeting the T β R1, Smad2, and/or Smad4 proteins for degradation.

4.2. Epithelial growth factor (EGF) signaling pathway

The EGF receptor (EGFR) subfamily of receptor

tyrosine kinases are cell-surface receptors, consisting of 4 homologous members: EGFR, ErbB2/HER2, ErbB3, and ErbB4/HER4 (32). EGFRs are overexpressed in multiple tumors, including breast cancer and lung cancer (33).

ErbB4 has three PY motifs that are the targets of several WW domain containing proteins, such as WWOX (34), YAP (35, 36) and AIP4/Itch (37). Recently, two independent studies suggest that WWP1 promotes ErbB4 protein ubiquitination and degradation (16, 38). The protein-protein interaction between WWP1 and ErbB4 is through the first and third WW domains of WWP1 and the second PY motif (PPAY) of ErbB4-CYT1 that overlaps with the docking site (YTPM) of PI3K (16). The phosphorylated tyrosine will activate the PI3K/AKT survival signaling pathway (39) while the non-phosphorylated PY motif may facilitate WWP1 and Itch binding. Thus, the tyrosine phosphorylation of ErbB4 may act as a binary switch for ErbB4's activity and stability. A similar regulation has been observed in RNA Polymerase II (13).

ErbB4 is well known to undergo a two-step proteolytic cleavage, during which a membrane-anchored fragment of 80 kDa is first produced and a soluble 80-kDa fragment is subsequently liberated (40). WWP1-mediated degradation is more efficient for the full-length ErbB4 protein and for the membrane-anchored fragment as compared to the soluble fragment (38). Unlike other EGFR subfamily members, the ErbB4/HER4 expression correlates with positive ER α , lower tumor grade, and a better prognosis (41, 42). Intriguingly, the expression levels of WWP1 and ErbB4 seem to be correlated in breast cancer cell lines (16).

Additionally, our previous study showed that

WWP1 overexpression also upregulated the EGFR and ErbB2 expression levels in MCF10A and MDA-MB-231 breast cell lines in a manner independent of E3 ligase activity (11). WWP1 depletion decreased the cell surface EGFR and ErbB2 levels. The regulation of EGFR and ErbB2 by WWP1 may partially rely on RNF11, a PY motif containing RING finger type E3 ligase promoting EGFR and ErbB2 protein degradation. The interaction between WWP1 and RNF11 is through the first and third WW domains of WWP1 and the PY motif of RNF11 (11).

EGFR is well known to undergo rapid endocytosis upon ligand stimulation. SPG20/Saprtin is necessary for efficient degradation of EGFR (43). Recently, two independent studies suggested that WWP1 interacts with SPG20 through WW/PY motifs (44, 45). SPG20 can be recruited to the endosome and to lipid droplets (44, 45). While WWP1 overexpression can promote SPG20 mono-ubiquitination, the mono-ubiquitination of endogenous SPG20 is not affected by WWP1 depletion (44, 45). Interestingly, SPG20 interacts with EPS15, an endocytosis protein also mono-ubiquitinated by WWP1 (3, 44).

Taken together, WWP1 may regulate the EGF signaling pathway by not only directly targeting ErbB4 for degradation but also indirectly inhibiting EGFR and ErbB2 endocytosis through other proteins, including RNF11, SPG20, and EPS15.

4.3. Krüppel-like factor (KLF) family

The KLF family members are well established zinc finger transcription factors that play critical roles in cell proliferation, survival, differentiation, and stemness (46). Both KLF5 and KLF2 have been implicated in cancers and cardiovascular diseases and reported to be WWP1 substrates.

KLF5 promotes cell proliferation, survival, and angiogenesis (47). KLF5 turns over rapidly through the ubiquitin-proteasome pathway (48). The KLF5 transactivation domain contains a PY motif that interacts with the WW domains of WWP1 (23). The E3 ligase activity of WWP1 is essential for WWP1 to target KLF5 for ubiquitination and degradation (23). WWP1 depletion increases the endogenous KLF5 protein levels in MCF10A, MCF7, and PC-3 (23, 49). As expected, the expression of WWP1 is negatively correlated with that of KLF5 in human breast cancer cell lines (49).

In contrast to KLF5, KLF2 does not have a PY motif. Zhang *et al.*, reported that WWP1 interacted with the inhibitory domain of KLF2, ubiquitinated KLF2, and targeted it for proteasomal degradation in an E3 ligase-independent manner (50). These findings suggest that WWP1 may facilitate KLF2 ubiquitination and degradation as an adaptor protein. Whether endogenous WWP1 regulates KLF2 has not been investigated.

4.4. p53 family

The p53 transcription factor family contains three members, p53, p63, and p73. p53 is a well-known tumor suppressor that responds to diverse cellular stresses and

induces cell cycle arrest, apoptosis, and senescence (51). p53 has been reported to be ubiquitinated by several E3 ligases, such as MDM2 (52), Pirh2 (53), and WWP1 (29).

Laine *et al.* reported that WWP1 interacts with the p53 DNA-binding domain. The proline-rich domain of p53 does not have a PY motif but increases the efficiency of protein-protein interaction (29). WWP1 ubiquitinated p53 in an E3 ligase dependent manner and increased the nuclear export of p53 (29). We demonstrated that WWP1 targeted p63 for ubiquitin-mediated protein degradation (15). Using different promoters, p63 can be expressed as Δ Np63 and TAp63 that have an opposite function in terms of apoptosis. The pro-survival Δ Np63 protein is the predominant p63 isotype in the prostate and breast (15). There are three isoforms (α , β , γ) for both Δ Np63 and TAp63. WWP1 interacts with p63 α through WW-PY motifs and targets p63 α for ubiquitin-mediated proteasomal degradation (15). In Δ Np63 positive breast cells, WWP1 depletion confers apoptosis resistance (15). Whether WWP1 targets the PY motif containing p73 for degradation has not been tested. Interestingly, WWP1 is induced by DNA damage agents in a p53 dependent manner (15). Thus, WWP1 is p53 downstream gene that can inhibit p53 family member activities through a feedback mechanism.

4.5. RUNX2

RUNX2 is a principle transcription factor for osteoblast differentiation and associates with tumor growth in the bone (54). RUNX2 is frequently overexpressed in invasive breast and prostate cancers (54). WWP1 uses Shn3 or Smad6 as adaptors to target RUNX2 for ubiquitination and degradation although RUNX2 itself has a PY motif (55, 56). Shn3 increases the protein interaction between WWP1 and RUNX2 (55). Mice lacking Shn3 (55) or Wwp1 display increased bone mass (personal communication with Dr. Lianping Xing at University of Rochester). Surprisingly, the expression levels of Wwp1/Shn3 and Runx2 increased during osteoblast differentiation (28). It was speculated that WWP1 only functions at the late stage of osteoblast differentiation, as RUNX2 must be downregulated in fully matured osteoblasts. WWP1 may play significant roles not only in osteoblast differentiation but also in tumor bone metastasis (personal communication with Dr. Lianping Xing).

5. WWP1 AND DISEASES

WWP1 is ubiquitously expressed in human tissues and participates in multiple physiological and pathological processes, such as cancers, infectious diseases, neurological diseases, and even aging.

5.1. Cancers

WWP1 appears to be a context dependent oncogene in cancers. The WWP1 mRNA and protein levels are frequently up-regulated in a subset of prostate and breast cancers (20, 21). Interestingly, the expression of WWP1 in breast tumors correlates with positive ER α and insulin-like growth factor 1 receptor (IGF-1R) statuses, which are good prognosis biomarkers in breast tumors. Indeed, Huu *et al.* found that tumors with low/absent WWP1 expression have

a worse prognosis than tumors with WWP1 expression (24). Similar results were observed in head and neck squamous cell carcinoma (57). In MCF10A breast cells, when WWP1 was knocked down by siRNA, the cells became more resistant to doxorubicin-induced apoptosis (15). This result suggests that WWP1 has a pro-apoptotic function in MCF10A. The WWP1 pro-apoptotic function can be attributed to targeting several pro-survival proteins, such as Δ Np63 (15) and KLF5 (23), for ubiquitin-mediated degradation. Consistently, the expression of WWP1 is negatively correlated with the expression of Δ Np63 (15) and KLF5 (49) in breast cancers.

In contrast to the WWP1 pro-apoptotic function in ER α -negative breast cells, WWP1 depletion in ER α -positive MCF7 and HCC1500 breast cancer cell lines suppressed cell proliferation and induced apoptosis (11, 21, 24, 27). Similar results were obtained in MDCK (1), PC-3 (20), and HCT116 cells (15). In addition, WWP1 overexpression promoted breast epithelial cell growth and anchorage-independent growth (24). WWP1 enhances cell proliferation and survival likely through both ubiquitin ligase-dependent and -independent activities. In MDCK cells, WWP1 suppressed the expression of T β R1, Smad2, and TGF β -induced PAI1 and JunB (1). In PC-3, WWP1 suppressed the expression of T β R1, Smad4, and the cell cycle dependent kinase inhibitor p15 (20). In HCT116, WWP1 targeted the TAp63 for degradation (15). In addition, WWP1 enhances MAPK signaling through decreasing the ErbB2 and EGFR turnover (11). Thus, WWP1 may have a context dependent role in cancer development. To conclusively sort this out, the physiological role of WWP1 needs to be elucidated in animal models.

5.2. Infectious diseases

Available evidence suggests that WWP1 may participate in viral internalization and budding. Galinier *et al.*, first reported that WWP1 interacted with the adenovirus penton base protein that has two PY motifs and is involved in viral internalization (58). It is becoming obvious that WWP1 promotes retrovirus particle assembly and release through binding to Gag. The Gag precursor protein is well known to drive the assembly and release of enveloped retroviruses. Many Gag late assembly function domains contain a PY motif (59). Heidecker *et al.*, reported that mutation of the PY motif severely decreased human T-cell leukemia virus type 1 (HTLV-1) virus budding (60). Chen *et al.*, suspected that Gag of avian retroviruses and LMP2 of the Epstein-Barr virus (EBV) may interact with WW domain containing proteins through their PY motifs (61). Consistently, *cis* expression of a WW domain in the Gag protein can block budding of Rous sarcoma virus (RSV) (62). These studies suggest that the Gag PY motif is essential for budding through recruiting the WW domain proteins.

WWP1 has been demonstrated to mono-ubiquitinate Gag proteins at a single lysine (44, 60) and mutation of the Gag ubiquitination site dramatically inhibited viral budding (63), suggesting that the Gag ubiquitination by WWP1 may play a role for viral budding.

This notion is supported by another independent study (59). Martin-Serrano *et al.* reported that the E3 ligase activity was required for WWP1 functions in this context (59). The HECT domains from WWP1, WWP2, or Itch recruited class E vacuolar protein-sorting (VPS) factors and ubiquitinated them for viral budding (59). Importantly, *trans* expression of dominant negative WWP1 (C2-WW or WW) proteins inhibited budding of HTLV-1, murine leukemia virus (MLV), and Ebola viruses (59, 60). These studies suggest that the WW domain polypeptide may be a potential therapeutic intervention for viral infection.

WWP1 may also be involved in innate immunity. *Wwp-1* deficient *C. elegans* are hypersensitive to killing by pore-forming toxins and bacterial pathogens *Pseudomonas aeruginosa* (64). *Wwp-1* may regulate innate immunity through antagonizing the DAF-2 insulin-like signaling pathway in *C. elegans*. Whether WWP1 plays a role in mammalian immunity is currently unknown.

5.3. Neurological diseases

Ubiquitination is well documented to play important roles in neurological diseases. WWP1 interacts with several proteins implicated in neurological diseases. Firstly, WWP1 interacts with Atrophin-1, a nuclear receptor corepressor containing PY motifs and a polyglutamine repeat (2). *Atrophin-1* was identified as a neurodegenerative disease gene because it is mutated in dentatorubral-pallidoluysian atrophy. Disease-causing mutations are expansions of a DNA triplet repeat leading to the expression of a protein with an extended stretch of glutamine residues that causes neuronal death (65). The functional consequences of the WWP1/Atrophin-1 interaction have not been explored yet. Secondly, WWP1 interacts with Nogo-A, a key inhibitor for central nervous system (CNS) regeneration (66). The Nogo-A protein contains a PY motif and localizes to the endoplasmic reticulum (ER). The protein solution structure of WWP1 WW4 domain with the Nogo-A PY motif peptide has been determined by NMR (66). Qin *et al.*, claimed that WWP1 ubiquitinated Nogo-A and regulated the Nogo-A protein level (66). It would be interesting to investigate whether WWP1 regulates CNS regeneration. Finally, WWP1 interacts with Saprtin/SPG20, a protein mutated in Troyer syndrome (44, 45, 67). The aforementioned studies suggest that WWP1 mono-ubiquitinates SPG20 and regulates its protein levels and subcellular localization (44, 45).

5.4. Aging and other diseases

In response to dietary restriction, *wwp-1* acts as a positive regulator of lifespan in E3 ubiquitin ligase-dependent manner in *C. elegans* (68). In contrast to three WWP ligases (WWP1, WWP2, and AIP4) in mammals, there is only one WWP E3 ligase in *C. elegans*. Depletion of *wwp-1* leads to abnormal embryogenesis and a lethal phenotype (18). Carrano *et al.*, demonstrated that loss of *wwp-1* functions by RNAi or mutation decreases lifespan. The *wwp-1* transgenic lines lived up to 20% longer than the controls (68). Chen *et al.*, confirmed that *wwp-1* mutations decreased lifespan in *C. elegans* (64). The possible molecular mechanism by which *wwp-1* participates in longevity regulation is that *wwp-1* is inhibited by the PDK-

1 kinase downstream of the DAF-2 insulin/IGF-1 signaling pathway in *C. elegans* (64). It would be interesting to elucidate the role and mechanistic of action of WWP1 in mammalian longevity.

WWP1 has also been reported to be responsible for chicken muscular dystrophy (69). A WWP1 missense mutation was identified in dystrophic chickens (69). In addition, WWP1 may be involved in bone diseases because it targets RUNX2 for degradation (55, 56).

6. CONCLUSIONS AND PERSPECTIVES

In summary, WWP1 is a WW domain containing HECT type ubiquitin E3 ligase. WWP1 targets a number of PY motif containing substrate proteins, including Smad2, KLF5, p63, ErbB4, RUNX2, RNF11, and SPG20 for ubiquitination. In addition, WWP1 may indirectly regulate other substrates without a PY motif through adaptors. WWP1 plays important roles in a variety of diseases, such as cancers, infectious diseases, neurological diseases, and aging.

In the future, *Wwp1* knock-out and tissue specific transgenic mouse models will be urgently required to elucidate the physiological and pathological functions of WWP1 in human diseases. Xenograft mouse models will be also useful to determine the role of WWP1 in cancer growth and metastasis. Whether the genetic and expression alterations of WWP1 can be used as biomarkers in cancers and other diseases should be studied in large cohorts of patients. Additionally, it is very important to dissect the distinct and redundant functions of the WWP1 E3 family members in order to design the most effective interventions. Furthermore, the mechanism of WWP1 regulation is still poorly understood. Finally, it would be interesting to develop small molecular inhibitors or peptides for treating WWP1-related diseases.

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Key Words: WWP1, WW domain, PY motif, ubiquitin E3 ligase, Ubiquitination, Cancer, Review

Send correspondence to: Ceshi Chen, The Center for Cell Biology and Cancer Research, Albany Medical College, 47 New Scotland Ave. Albany, NY, USA, 12208, Tel: 518-262-2936. E-mail: chenc@mail.amc.edu

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WWP1 regulates cell apoptosis through targeting p63 for ubiquitin-mediated proteasomal degradation

Yi Li, Zhongmei Zhou, Ceshi Chen*

The Center for Cell Biology and Cancer Research, MS355, Albany Medical College, 47 New Scotland Ave. Albany, NY, 12208

E-mail: chenc@mail.amc.edu

WWP1, a HECT domain-containing protein ubiquitin E3 ligase, has previously shown to be frequently amplified and overexpressed in a subset of prostate and breast cancer. However, the role and mechanism of WWP1 in human cancer is still largely unknown. Here, we show that WWP1 binds, ubiquitinates, and targets p63 for proteasomal degradation. p63, a member of the p53 family of transcription factors, plays an important role in tumor development by regulating cell apoptosis. Using alternative promoters, p63 can be expressed as TAp63 or Δ Np63. Increasing evidences suggesting that TAp63 sensitizes cell to apoptosis but Δ Np63 plays an opposite role. It is already known that p63 proteins are degraded through the ubiquitin-proteasome pathway. We found that the protein-protein interaction occurs between the PY motif of p63 and the WW domains of WWP1, mainly the first WW domain. The Y449F point mutation (disruption of the PY motif) in Δ Np63 α significantly abrogates the interaction with WWP1 and protects the p63 protein from degradation. Interestingly, the endogenous p63 expression level, especially Δ Np63 α , is reversely correlated with the endogenous WWP1 expression level in a panel of prostate and breast cancer cell lines. WWP1 Knockdown increases the endogenous level of Δ Np63 α in immortalized MCF10A and 184B5 cell lines and TAp63 α in HCT116 colon cancer cell line. Consistently, WWP1 regulates chemotherapeutic agents induced cell apoptosis. These data suggest that WWP1 has an important role in regulating p63 protein level and therefore affects drug resistance of cancer cells.

Key words: WWP1, E3 ligase, p63, ubiquitin, degradation, apoptosis, cancer

Grant support: Department of Defense Prostate Cancer Program and Susan G. Komen Breast Cancer Foundation



Category: Cellular And Molecular Biology 5

Session Title: Oncogenic Receptor Pathways

#140 WWP1 promotes cell proliferation through blocking RNF11 mediated Erbb2 and EGFR

downregulation. Ceshi Chen¹, Yi Li¹, Zhongmei Zhou¹, Arun K. Seth². ¹Albany Medical College, Albany, NY; ²Sunnybrook and Women's College Health Science Center, Toronto, ON, Canada.

The WW domain containing E3 ubiquitin protein ligase 1, WWP1, is an HECT type E3 ligase frequently overexpressed in human prostate and breast cancers due to the chromosomal amplification at 8q21. Previous studies suggest that WWP1 promotes cell proliferation and survival; however, the mechanism of WWP1 action is still poorly understood. Here, we showed that WWP1 upregulates ErbB2 and EGFR in multiple breast and prostate cell lines. WWP1 forms a protein complex with RNF11, a negative regulator of ErbB2 and EGFR. The protein-protein interaction is through the first or the third WW domain of WWP1 and the PY motif of RNF11. WWP1 does not target RNF11 for degradation although WWP1 ubiquitinates RNF11. Importantly, inhibition of RNF11 can rescue WWP1 siRNA-induced ErbB2 and EGFR downregulation and growth arrest. Finally, we demonstrated that RNF11 is co-overexpressed with WWP1 in human prostate and breast cancer cell lines. These findings suggest that WWP1 may promote cell proliferation through suppressing RNF11-mediated ErbB2 and EGFR downregulation.

Citation Format

Chen C, Li Y, Zhou Z, Seth AK. WWP1 promotes cell proliferation through blocking RNF11 mediated Erbb2 and EGFR downregulation [abstract]. In: Proceedings of the 99th Annual Meeting of the American Association for Cancer Research; 2008 Apr 12-16; San Diego, CA. Philadelphia (PA): AACR; 2008. Abstract nr 140.

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Category: Cellular And Molecular Biology 21

Session Title: New Oncogene And Tumor Suppressor Pathways

#1790 HECTD3: A new e3 ligase upregulated in breast and prostate cancer. Yi Li, Zhongmei Zhou, Ceshi Chen. Albany Medical College, Albany, NY.

Chromosomal region 1p34 undergoes frequent cytogenetic alterations in many types of cancer. The HECTD3 gene, encoding an HECT domain-containing E3 ubiquitin ligase, is located at the 1p34.1 region. HECTD3 mRNA is highly expressed in salivary gland, liver, thyroid, but low in normal breast and prostate tissues. To determine the role of HECTD3 in breast and prostate cancers, we analyzed the gene dosage and expression level of HECTD3 in a variety of breast and prostate tumor cell lines. Using real-time RT-PCR and Northern blot analysis, we found that the HECTD3 mRNA level was significantly up-regulated in 63.3% (19/30) of breast and 62.5% (20/32) of prostate cancer cell lines. HECTD3 levels were not correlated with ER or PR status ($p = 0.705$ and 1.0 , respectively) in breast cancer cell lines. An increase in HECTD3 gene copy number was detected in 48% (14/30, $p = 0.0051$) and 22% (4/18, $p = 0.0051$) of breast and prostate cancer cell lines, respectively. Additionally, HECTD3 mRNA levels were consistently elevated in all stages of breast cancer: stage I (9/11), IIa (7/8), IIb (4/6), IIIa (7/8), and IV (3/4). In contrast, HECTD3 was over-expressed in approximately 40% of stage II (7/19) and stage III (3/8) prostate tumors. RNAi-mediated HECTD3 knockdown induced cell growth arrest in PC-3 (prostate) and HCC1500 (breast) cancer cell lines. Together, these results suggest that HECTD3 is a novel oncogenic E3 ligase upregulated in human breast and prostate cancer.

Citation Format

Li Y, Zhou Z, Chen C. HECTD3: A new e3 ligase upregulated in breast and prostate cancer [abstract]. In: Proceedings of the 99th Annual Meeting of the American Association for Cancer Research; 2008 Apr 12-16; San Diego, CA. Philadelphia (PA): AACR; 2008. Abstract nr 1790.

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A 9-amino acid Motif at KLF5 N-terminus Contributes to WWP1 E3 Ubiquitin Ligase Mediated KLF5 Protein Degradation

Hanqiu Zheng, Zhongmei Zhou, and Ceshi Chen*

Center for Cell Biology and Cancer Research, Albany Medical College, Albany, NY 12208

*E-mail: chenc@mail.amc.edu

Kruppel-like factor (KLF5) is a zinc finger transcription factor, which plays important roles in cell proliferation, cell cycle, differentiation and angiogenesis. The KLF5 protein undergoes multiple post-translational modification, including phosphorylation, acetylation, and ubiquitination. In our previous studies, we demonstrated that the WWP1 E3 ligase interacts with KLF5 through the WW/PY motifs, ubiquitinates KLF5, and targets KLF5 for proteasomal degradation. Additionally, we recently demonstrated that the N-terminal KLF5 without the PY motif is still degraded through proteasome. To map the N-terminal motif responsible for KLF5 degradation, we made a series of C-terminal truncated KLF5 constructs and found that a 9-amino acid motif at KLF5 N-terminus is essential for WWP1-mediated KLF5 degradation. Interestingly, deletion of this motif in KLF5 does not affect the protein-protein interaction between KLF5 and WWP1 but inhibits KLF5 ubiquitination by WWP1. These findings suggest that the PY motif is essential but not sufficient for WWP1-mediated KLF5 ubiquitination and degradation. The discovery of this new KLF5 destruction motif may help us better understand the KLF5 regulatory mechanism and provide a new strategy to intervene KLF5 activity for cancer therapy.

Key words: KLF5, WWP1, ubiquitin, degradation

Grant support: Department of Defense Prostate Cancer Program and Susan G. Komen Breast Cancer Foundation



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WW domain containing E3 ubiquitin protein ligase 1 (WWP1) expression in breast cancer and its correlation with estrogen receptor (ER) and insulin-like growth factor receptor 1 (IGF-1R) status

Author Block: J. S. Ross, E. Slodkowska, C. E. Sheehan, Z. Zhou, C. B. Sheehan, c. chen; Albany Medical College, Albany, NY

Abstract:

Background:

WWP1, a HECT type E3 ubiquitin ligase, is frequently amplified and overexpressed in breast cancer and may associated with positive ER status. IGF-1R is a tyrosine kinase growth factor receptor that has been linked to prognosis in a variety of malignancies. Activation of IGF-1R by autocrine, paracrine and endocrine stimulation via exposure to its activating ligand, insulin, leads to cell growth.

Methods:

Formalin-fixed, paraffin-embedded tissue sections from 122 cases of invasive mammary carcinoma (90 ductal carcinomas (IDC) and 32 lobular carcinomas (ILC) were immunostained by automated methods (Ventana Medical Systems Inc., Tucson, AZ) using mouse anti-human WWP1 (Novus Biologicals, Littleton, CO) and mouse anti-human IGF-1R antibody (sc-462; Santa Cruz Biotechnology, Santa Cruz, CA). Cytoplasmic immunoreactivity was semiquantitatively scored based on staining intensity and distribution for each protein and the results were correlated with morphologic and prognostic variables. The correlations between WWP1 and ER/IGF-1R were also analyzed in a panel of breast cancer cell lines by Western blot. WWP1 knockdown studies were used to test WWP1 regulation of ER and IGF-1R in ER positive breast cancer cell lines.

Results:

The adjacent benign epithelium was essentially negative for both proteins. Cytoplasmic WWP-1 immunoreactivity was observed in 53/122 (43%) tumors and showed a positive correlation with ER status ($p=0.038$). Cytoplasmic IGF-1R positivity was observed in 66/122 (54%) tumors and correlated with tumor subtype (61% IDC vs. 34% ILC, $p=0.011$) and ER status within the IDC subgroup ($p=0.036$). There was a significant co-expression of both proteins ($p=0.001$). The positive correlations between WWP-1 and ER/IGF-1R were also observed in a panel of breast cancer cell lines assessed by Western blot. WWP1 knockdown decreased the expression levels of both ER and IGF-1R in MCF7 and T47D cell lines.

Conclusions:

WWP-1 and IGF-1R proteins are overexpressed and are associated with each other and the ER+ phenotype in breast carcinoma. Further development of WWP-1 and IGF-1R as biomarkers for breast cancer management appears warranted.

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145 A genome wide RNAi screen to identify essential transcription factors for the proteasome pathway in *Drosophila*

Anne Beskow and Patrick Young

While a clear regulatory pathway has been demonstrated in budding yeast, the mechanisms that regulate metazoan proteasomes are poorly understood. As an initial step to understand transcriptional induction of *Drosophila* proteasome genes, we have attempted to find key transcription factors for the proteasome pathway using a genome wide RNAi screening method. *Drosophila* S2 cells were transiently transfected with the proteasome UFD substrate Ub^{G76V}GFP to identify transcription factors that when knocked down allow general transcription to still occur but target the UPS pathway and thereby serve as an initial screen for genes products that are essential for its degradation. The screen consisted of the complete set of *Drosophila melanogaster* genes that encode 993 known or predicted transcription factors. Out of these, manual scoring for GFP accumulation revealed that 60 knockdowns had increased levels of Ub^{G76V}GFP. A range of stabilizations was obtained varying from barely detectable increases, majority of cases, to strong stabilization of the proteasome substrate reporters. Of special interest were three genes, since knock down of these showed the strongest stabilization of the UFD substrate. These were FOXO, cnc and maf-S. The cnc transcription factor is the *Drosophila* homolog of Nrf2. The maf-S forms a heterodimer transcription factor with cnc. Both FOXO and cnc/maf-S recently have been associated with proteasome induction in mammalian cells (Zhao et al. 2007) and (Kwak et al. 2003). We are currently carrying out a second round of RNA interference experiments to confirm our positive list and have initiated studies to determine which specific proteasome genes are transcriptionally regulated by our identified *Drosophila* candidates.

146 The E2 ubiquitin-conjugating enzyme Ube2S is required for mitotic spindle checkpoint function in human cells

Scott Crowder, Wayne Lang, Charleen Rayl, Gerald Uy, Amy Ondris, Jianing Huang, Yasumichi Hitoshi, Donald G. Payan, and Yonchu Jenkins

Rigel Pharmaceuticals, Inc. South San Francisco, CA 94080

Many enzymes of the ubiquitin modification pathway are employed by cells to monitor critical checkpoints in the cell cycle, control proliferation, monitor DNA damage and the integrity of the mitotic spindle. These enzymes include the E3 ubiquitin ligase of the anaphase promoting complex (APC) in concert with E2 ubiquitin-conjugating enzymes such as UbcH10. Cell cycle checkpoints are often defective in tumor-derived cells and may be key determinants of oncogenesis. We report that an E2 ubiquitin-conjugating enzyme, Ube2S, is required for spindle checkpoint integrity in HeLa cells. Knockdown of Ube2S protein levels allows cells to escape mitotic arrest induced by nocodazole treatment. This effect is phenocopied by expression of Ube2S C95S, a point mutant that is catalytically impaired. Consistent with these findings, Ube2S expression and localization is cell-cycle dependent, upregulated in a number of tumors, and was recently reported to be associated with the APC (Steen et al., *Proc Nat Acad Sci USA*, 2008). We also characterize the *in vitro* autoubiquitylation activity of both wild-type and mutant Ube2S proteins and identify residues responsible for a monoubiquitylation activity of Ube2S. *In vitro*, disruption of this monoubiquitylation site results in a dramatic switch from low molecular weight ubiquitin-conjugated Ube2S proteins to higher molecular weight polyubiquitin-conjugated Ube2S proteins. Expression of this Ube2S point mutant in HeLa cells also enables cells to override nocodazole-induced spindle checkpoint activation, suggesting that the unique Ube2S autoubiquitylation properties observed *in vitro* may be crucial for regulating Ube2S spindle checkpoint activity *in vivo*.

147 Chemiluminescent immunoassay for the quantitative determination of WWP-1 E3 ubiquitin ligase

Lothar Goretzki, Jue Wang, and Mo Saedi, EMD Chemicals, Inc. (Calbiochem), 10394 Pacific Center Court, San Diego, CA92121.

Accumulating evidence suggests that E3 ubiquitin ligases play important roles in cancer development. The WWP1 E3 ubiquitin ligase belongs to Nedd4-like family. All members of the Nedd4-family are HECT type E3 ligases with three functional domains: an N-terminal C2 domain for membrane binding, a central region containing WW domains for protein-protein interaction, and a C-terminal HECT domain for ubiquitin protein ligation. The WWP1 gene maps to 8q21, a region that frequently displays a gain of copy number in human cancers, including prostate and breast cancer. In agreement with the reported gene copy number gain, WWP1 expression is elevated at the mRNA and protein levels in about 60% of prostate and breast cancer specimen. Therefore, the WWP1 gene could be an oncogene in prostate and breast cancer. It has been shown that WWP1 negatively regulates the TGF β tumor suppressor pathway by mediating the ubiquitination and degradation of multiple components of the pathway, and subsequently promotes cell proliferation.

An immunoassay for the quantification of WWP1 was developed and evaluated for its use with various tumor cell extracts. The detection limit of the WWP1 immunoassay is 0.05 ng/ml. High levels of WWP1 protein were detected in hormone-independent prostate and breast cancer cells, DU145 (31 ng/mg total protein) and SK-BR-3 (38 ng/mg total protein), respectively.

In conclusion, WWP1 could serve as a novel biomarker and therapeutic target in the detection and treatment of prostate and breast cancer. The presented immunoassay helps to define more precisely the significance of WWP1 as an oncogenic factor in prostate and breast cancer.

148 Overexpression Of The Hect Type E3 Ubiquitin Ligase Wwp1 Is Associated With The Estrogen Receptor And Insulin-Like Growth Factor Receptor 1 (Igf-1r) In Breast CarcinomaCeshi Chen^{1*}, Zhongmei Zhou¹, Christine E. Sheehan², Elzbieta Slodkowska², Christopher B. Sheehan², Ann Boguniewicz², Jeffrey S. Ross²¹The Center for Cell Biology and Cancer Research, ²The Department of Pathology and Laboratory Medicine, Albany Medical College, 47 New Scotland Ave. Albany, NY, 12208*Correspondence should be addressed to: chenc@mail.amc.edu

WWP1, a HECT type E3 ubiquitin ligase frequently amplified and overexpressed in breast cancer, has the potential to become a useful clinical biomarker and therapeutic target in breast cancer. Here, we performed immunohistochemical staining in formalin-fixed and paraffin-embedded tissue sections from 187 cases of primary invasive mammary carcinoma (137 ductal carcinomas (IDC) and 50 lobular carcinomas (ILC) by using a monoclonal anti-WWP1 antibody. The adjacent benign epithelium was essentially negative for WWP1. Cytoplasmic WWP1 immunoreactivity was observed in 76/187 (40.6%) tumors and showed a positive correlation with ER (p=0.05) and IGF-1R proteins (p=0.001) in this cohort. The positive correlations between WWP1 and ER/IGF-1R were also observed in a panel of 12 breast cancer cell lines by Western blot. Interestingly, the ER levels are decreased when WWP1 is silenced in ER positive MCF7 and T47D breast cancer cell lines. Finally, WWP1 ablation additively inhibits cell proliferation with tamoxifen in MCF7 and T47D, as measured by ³H-thymidine incorporation assays. These findings suggest that WWP1 may play an important role in ER positive breast cancer.

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Kruppel-like factor 5 promotes breast cancer proliferation through activating fibroblast growth factor-binding protein transcription.

Zheng H, Zhou Z, Chen C, Albany Medical College, Albany, NY

Breast cancer is the second leading cause of cancer death in U.S. women. The estrogen receptor negative basal breast cancer is usually invasive and the prognosis is poor. Kruppel-like Factor 5 (KLF5) is a zinc-finger transcription factor promoting cell proliferation, survival, cell cycle, and angiogenesis. High expression level of KLF5 has been shown to be associated with shorter breast cancer patient survival time. In our previous microarray studies, fibroblast growth factor binding protein (FGF-BP) is suggested to be one of the KLF5's downstream target genes in the TSU-Pr1 bladder cancer cell line. FGF-BP has been appreciated to be overexpressed in breast tumors and promotes tumorigenesis. To test whether KLF5 promotes cell proliferation through inducing FGF-BP gene transcription in breast cells, we first examined the KLF5 and FGF-BP protein levels in a panel of breast cell lines and found that KLF5 and FGF-BP are co-expressed in ER-negative basal breast cell lines. Over-expression of KLF5 by adenovirus dramatically increased the FGF-BP mRNA and protein expression in MCF7 and BT474. In contrast, KLF5 knockdown decreased the FGF-BP mRNA and protein expression in HCC1937, BT20, and SW527. Importantly, we found that FGF-BP, like KLF5, promotes cell proliferation in MCF10A, SW527 and TSU-Pr1 cells by H3-thymidine incorporation assay. Furthermore, we demonstrated that KLF5 activates the FGF-BP promoter through a putative KLF5 binding site

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by luciferase reporter assays. We further showed that KLF5 binds to the FGF-BP promoter *in vitro* by oligo pull down assays and *in vivo* by chromatin immunoprecipitation (CHIP) assays. These findings suggest that KLF5 may promote breast cancer cell proliferation through directly activating the FGF-BP mRNA transcription. This study will yield valuable insights into the mechanism of KLF5 induced cancer pathogenesis, and will result in useful diagnostic and therapeutic targets.

Kruppel-like transcription factor 5 promotes breast cell survival through pERK-mediated MKP-1 protein stabilization.

Liu B, Zhou Z, Chen C. Albany Medical Center, Albany, NY

Kruppel-like Factor 5 (KLF5) is a transcription factor promoting cell survival and tumorigenesis in multiple cancers. High level of KLF5 mRNA is associated with a shorter survival for breast cancer patients. However, the role of KLF5 and mechanism of KLF5 actions in breast cancer remain unclear. In this study, we found that KLF5 knockdown by siRNA in two basal type breast cell lines MCF10A and BT20 induces apoptosis, as indicated by loss of cell viability and cleaved PARP and caspase 3. Interestingly, a survival phosphatase, MKP-1, is downregulated at protein level by KLF5 ablation. Consistently, KLF5 over-expression increases the MKP-1 protein expression. However, MKP-1 is not a KLF5 direct target gene because the MKP-1 mRNA level is not regulated by KLF5. By the cycloheximide chase assays, we found that KLF5 decreases the MKP-1 protein degradation. However, KLF5 does not decrease the MKP-1 E3 ubiquitin ligase SKP2 expression. The ERK inhibitor U0126 specifically blocks the KLF5 induced MKP-1, suggesting that KLF5 up-regulates MKP-1 through activating ERK signaling. Finally, we demonstrated that MKP-1 over-expression blocks KLF5 knockdown induced apoptosis in MCF10A cells. These findings suggest that KLF5 is a survival factor which promotes breast cell survival partially through pERK-mediated MKP-1 stabilization. The KLF5-pERK-MKP-1 signaling axis may provide new therapeutic targets for invasive breast cancer.



Title: E3 Ubiquitin Ligases as Novel Molecular Targets in Breast Cancer

Dr. Ceshi Chen

Assistant Professor

The Center for Cell Biology and Cancer Research

Albany Medical College

Albany, NY

USA

Abstract

Protein ubiquitination is an important posttranslational modification that regulates a multitude of cancer related cellular processes, including cell growth and death. Protein ubiquitination is typically sequentially mediated by three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). The E3 controls substrate specificity. In human beings, there are more than 600 E3s. Interestingly, many E3s undergo frequent genetic and expression aberrations and function as either tumor suppressors (i.e., BRCA1) or oncoproteins (i.e., Mdm2 and Skp2) in breast cancer. Several specific and effective small molecular inhibitors of Mdm2 have been reported to have a substantial p53-dependent anti-tumor effect *in vivo*. Therefore, oncogenic E3s are promising molecules for cancer target therapy. Our long term goal is to identify cancer related E3s as molecular targets and to improve cancer prevention, diagnosis, and therapy. We have recently demonstrated that the WW domain containing E3 ubiquitin protein ligase 1 (WWP1) gene is frequently amplified and overexpressed in estrogen receptor (ER) positive breast cancer. Inhibition of WWP1 alone by siRNA or dominant negative WWP1 induces apoptosis in ER positive breast cancer cell lines MCF7 and HCC1500 primarily through activation of caspase 8. The WWP1 siRNA induced apoptosis in MCF7 can be completely rescued by overexpression of wild type WWP1 but not the E3 ligase inactive WWP1C890A. Additionally, we found that inhibition of WWP1 reduces colony formation in soft agar and tumor growth in nude mice. Furthermore, we demonstrated that inhibition of WWP1 can be combined with tamoxifen or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) to suppress breast cancer cell proliferation and survival. Finally, we identified several WWP1 direct substrates including p63, KLF5, and ErbB4 as well as indirect targets including ER, EGFR, and HER2. These findings suggest that WWP1 is a novel molecular target in ER positive breast cancer.

The research is supported by U.S. Army, a BCRP Concept Award, a PCRP Idea Award, and an American Cancer Society Research Scholar Award.

Biography

Upon completion of his doctorate in Shanghai Research Center of Biotechnology, Chinese Academy of Sciences in 1999, he accepted his postdoctoral training at the University of Virginia where he conducted cancer genetic and cancer biology research. This led to the cloning of the KLF5 gene linked to prostate and breast cancer. In 2002, he moved to Emory University at Atlanta where Dr. Chen discovered that WWP1 E3 ligase ubiquitinates KLF5 and targets it for proteasomal degradation. He was promoted to Instructor in 2004. Dr. Chen joined the faculty of the Center for Cell Biology and Cancer Research at Albany Medical College (AMC) in 2006 where he is an Assistant Professor. At AMC, Dr. Chen has been interested in the role of several protein ubiquitin E3 ligases in breast cancer. It is expected that his work will lead to development of novel therapeutic targets. He published 17 first-author or corresponding author papers in prestigious journals such as Cell Death & Differentiation, Oncogene, JBC, and Am J Path. He has been reviewers for prestigious funding agents such as DoD, Komen for the Cure, and National Science Foundation of China and many prestigious journals including Cancer Research, Oncogene, and Cell Death & Differentiation.

Metastasis Studies. In the group that received buffer alone, 7 of 9 animals (78%) had large lesions either in bone or soft tissues. However, in the group that received the Ad.sT β RfC, only 2 of 9 animals (22%) developed tumors by day 45, indicating significant inhibition of metastases. The Ad.sT β RfC treated group had high levels ($> 300 \mu\text{g/ml}$) of sTGF β RIIFc in the blood, as well as viral replication in the tumors, suggesting that both viral replication and sTGF β RIIFc production are critical for the anti-tumor response of Ad.sT β RfC.

CONCLUSIONS: Data presented here shows that it is feasible to create an oncolytic adenovirus that can induce tumor cell destruction and simultaneously inhibit TGF β signaling. More importantly, the systemic administration of Ad.sT β RfC can inhibit metastases, suggesting that Ad.sT β RfC can be developed as an anti-tumor agent for the treatment of prostate cancer metastases.

Source of Funding: Funded by the Kovler Family Foundation, Mr. and Mrs. Richard Hulina, Mr. Jimmie Alford and Ms. Maree Bullock, and an anonymous donor.

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PEMPA1 PROMOTES PROSTATE CELL PROLIFERATION THROUGH DOWN-REGULATING P21WAF1/CIP1

Rong Liu*, Ceshi Chen, Albany, NY

INTRODUCTION AND OBJECTIVES: PMEPA1, prostate transmembrane protein, androgen induced 1, was originally identified as an androgen-induced gene. PMEPA1 has been suggested to be a tumor suppressor in prostate cancer because the forced over-expression of PMEPA decreases colony formation in several prostate cancer (PC) cell lines. However, a PC-3 clone with a higher level of PMEPA1 has been shown to grow faster in nude mice. Additionally, PMEPA1 has been reported to be over-expressed in multiple solid tumors, including breast cancer, ovarian cancer, renal cell carcinoma, and colon cancer. Thus, the physiological function of PMEPA1 in prostate cancer is controversial and unclear.

METHODS: Prostate epithelial cell lines (PC-3 and RWPE1) were transiently transfected with siRNA targeting PMEPA1. The gene expression at both RNA and protein levels, cell proliferation, and cell cycle were analyzed by qRT-PCR, Western blotting, the 3H-thymidine incorporation assay, and flow cytometry. A lentivirus based short hairpin (sh) RNA expression system was used to establish PMEPA1 stable knockdown populations in PC-3 cells. Luciferase shRNA was used as control. These stable PC-3 populations were used for in vivo tumorigenesis analysis in SCID hairless outbred (SHO) mice.

RESULTS: In our studies, we found that inhibition of endogenous PMEPA1 suppresses cell proliferation and cell cycle G1/S progression in RWPE1 and PC-3, but not in androgen receptor (AR) positive prostate cancer cell line 22Rv1 and LNCaP. PMEPA1 knockdown induces the expression of cyclin-dependent kinase inhibitor (CKI) p21Waf1/Cip1 at mRNA and protein levels. Furthermore, p21 knockdown can completely rescue PMEPA1 knockdown induced cell growth inhibition. Consistently, stable depletion of PMEPA1 in PC-3 prostate cancer cell inhibited tumorigenesis in SHO mice.

CONCLUSIONS: These findings support the hypothesis that PMEPA1 promotes androgen refractory prostate cancer cell proliferation through inhibiting the expression of p21Waf1/Cip1. The PMEPA1 might be developed as a molecular target for androgen refractory prostate cancer prevention, diagnosis, and therapy.

Source of Funding: AUA Foundation, DoD

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SUPPRESSION OF PROSTATE CANCER GROWTH BY A NEUTRALIZING ANTI-TRANSFORMING GROWTH FACTOR-BETA (TGF- β) ANTIBODY 1D11

Qiang Zhang*, Lin Chen, Brian Helfand, Norm Smith, Chicago, IL; Julie Zhu, Boston, MA; Borko Javonovic, Chicago, IL; Yinglu Guo, Beijing, China, People's Republic of; Chung Lee, Chicago, IL

INTRODUCTION AND OBJECTIVES: Transforming growth factor- β (TGF- β) is a potent immunosuppressant molecule. In previous studies, we showed that over-production of TGF- β by prostate cancer derived cells results in evasion of host immune surveillance and tumor progression. In this study, we explored the potential of immunotherapy for prostate cancer by a specific neutralizing anti-TGF- β antibody 1D11.

METHODS: We have obtained an expression vector with a triple fusion protein, containing HSV1-tk-GFP-luciferase (SFG-nTGL) from Dr. Vladimir Ponomarev of the Memorial Sloan Kettering Cancer Center. Mouse prostate cancer TRAMP-C2 cells are transfected with this reporter gene, designated as TRAMP-luciferase. This new approach will allow us to monitor tumor load in intact animals and the effects of therapy through a noninvasive bioluminescence imaging technology. The study was initiated using the approach of subcutaneous (sc) injection of TRAMP-luciferase cells (5×10^6 cells) into the right flank region of 30 C57BL/6 mice. Animals are randomly assigned to three groups following with intraperitoneal injection of a specific neutralizing anti-TGF- β antibody 1D11 or control antibody 13C4 respectively at the time of tumor cell injection. Group 1: 1D11 (50 mg/kg every 3 days); Group 2: 13C4 (50 mg/kg every 3 days), and Group 3: no treatment control. All the mice were killed after 15 time injection of antibodies. The size and weight of tumor, weight of prostate and spleen were measured.

RESULTS: In the group treated with 1D11 1 of 10 mice were free of tumor, and all the mice in 13C4 treatment group and control group have tumors. The average tumor weight and volume in the remaining 9 mice in 1D11 group was 5.3g and 6.85 cm² respectively which is almost 2-5 folds less than that of animals in 13C4 treatment group (15.8mg and 12.85 mm²) and no treatment group (31.4mg and 23.39 mm²) ($P < 0.01$). There is no significant difference on the weight of prostate and there is no significant metastasis on all the groups by the monitor of bioluminescence imaging. Interestingly, upon treatment with 1D11, the weight of spleen (101 mg) increased when compared to another two groups 76.4 mg and 80.4 mg respectively.

CONCLUSIONS: The data indicated that neutralization of tumor secreted TGF- β by 1D11 may induce the host immune response which could be helpful for recovery of host anti-tumor immune response inhibited by TGF- β . The inhibition of TGF- β then results in the suppression of prostate cancer growth. This study may lead to the development of effective antitumor therapeutic strategies in the near future.

Source of Funding: This study was supported in part by grants from the American Cancer Society, Illinois (#08-22), Department of Defense (W81XWH-09-1-0311), Portes Center/Institute of Medicine of Chicago (QZ), American Cancer Society Institutional Research Grant (ACS-IRG 93-037-12), American Urological Association Foundation (AUA Foundation, QZ), National Cancer Institute (P50CA090386), a grant from the Genzyme Corporation, and a gift from Mr. Fred L. Turner.



Title: Targeting the KLF5 Transcription Factor for Ubiquitin Proteasome Degradation in Breast Cancer

Dr. Ceshi Chen

Abstract

Protein ubiquitination is an important posttranslational modification. Protein ubiquitination is typically sequentially mediated by three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). The E3 controls substrate specificity. Interestingly, many E3s undergo frequent genetic and expression aberrations and function as either tumor suppressors (i.e., SCF^{Fbw7}) or oncoproteins (i.e., Mdm2 and SCF^{Skp2}) in breast cancer. Several specific and effective small molecular inhibitors of Mdm2 and CRL have been reported to have a substantial anti-tumor effect *in vivo*. Therefore, oncogenic E3s are promising molecules for cancer target therapy. The KLF5 transcription factor is overexpressed in ER negative basal type breast cancer and promotes breast cell proliferation and tumorigenesis through upregulating its target gene *FGF-BP*. Our previous studies suggest that the KLF5 protein turns over rapidly by the WWP1 E3 ligase through the ubiquitin proteasome pathway. In this study, we demonstrate that the Skp1-Cul1-Fbw7 E3 ubiquitin ligase complex (SCF^{Fbw7}) targets KLF5 for ubiquitin-mediated degradation in a KLF5 phosphorylation dependent manner. Fbw7 interacts with KLF5 through the WD40 repeats of Fbw7 and a CPD motif (³⁰³SPPSS) of KLF5 and promotes KLF5 ubiquitination and degradation. Both the F-box and WD40 repeats of Fbw7 are required for KLF5 ubiquitination and degradation. Mutation of the critical S303 residue in the KLF5 CPD motif abolishes the protein interaction, ubiquitination, and degradation by Fbw7. Using a KLF5 phosphorylation specific antibody and the *in vitro* kinase assay, we demonstrate that the KLF5 S303 can be phosphorylated by GSK3 β . Inhibition of GSK3 β activity by LiCl delays the KLF5 degradation. Importantly, inactivation of endogenous Fbw7 by gene knockout, knockdown, or mutation remarkably increases the endogenous KLF5 protein abundances through blocking its degradation. The endogenous Fbw7 isoforms (α , β and γ) can promote endogenous KLF5 degradation in a cell line dependent manner. Finally, we found that endogenous Fbw7 suppresses the *FGF-BP* gene expression and breast cell proliferation through targeting KLF5 for degradation. These findings suggest that the Fbw7 tumor suppressor inhibit breast cancer cell proliferation at least partially through targeting KLF5 for ubiquitin-mediated degradation. This new regulatory mechanism of KLF5 degradation may result in useful diagnostic and therapeutic targets for breast cancer and other cancers.

The research is supported by an American Cancer Society Research Scholar Award and a DoD IDEA Award.

Biography

Upon completion of his doctorate in Shanghai Research Center of Biotechnology, Chinese Academy of Sciences in 1999, he accepted his postdoctoral training at the University of Virginia where he conducted cancer genetic and cancer biology research. This led to the cloning of the KLF5 gene linked to prostate and breast cancer. In 2002, he moved to Emory University at Atlanta where Dr. Chen discovered that WWP1 E3 ligase ubiquitinates KLF5 and targets it for proteasomal degradation. He was promoted to Instructor in 2004. Dr. Chen joined the faculty of the Center for Cell Biology and Cancer Research at Albany Medical College (AMC) in 2006 where he is an Assistant Professor. Dr. Chen was promoted to Associate professor in 2009. At AMC, Dr. Chen has been interested in the role of several protein ubiquitin E3 ligases in breast cancer. It is expected that his work will lead to development of novel therapeutic targets. He published 22 first-author or corresponding author papers/reviews in prestigious journals such as *Cell Death & Differentiation*, *Oncogene*, *JBC*, and *Am J Path*. He has been reviewers for prestigious funding agents such as DoD, Komen for the Cure, and Natural National Science Foundation of China and many prestigious journals including *Cancer Research*, *Oncogene* and *Cell Death & Differentiation*.

Dr. Ceshi Chen

Associate Professor

Center for Cell Biology and Cancer Research

Albany Medical College

USA

tance to tumor formation seen in this study may provide important insight into regulatory mechanisms present in bronchial epithelial cells routinely exposed to environmental carcinogens.

#3149 4E-BP1 loss of function in pancreatic carcinogenesis. Rania Azar, Christiane Susini, Corinne Bousquet, Stephane Pyronnet. *INSERM U858, Toulouse, France.*

Inactivation of the transcription factor Smad4/DPC4 gene is a frequent event that correlates with aggressiveness of pancreatic adenocarcinomas. We have previously shown that the stress-inducible translational inhibitor 4E-BP1 is a target of Smad4 essential for TGF β -mediated inhibition of cell proliferation. Here, we show that 4E-BP1 expression parallels that of Smad4 through the graded stages of pancreatic cancer progression. 4E-BP1 amount transiently increases in human PanIN-1 and PanIN-2 lesions, but is dramatically diminished in PanIN-3 and invasive carcinomas. A similar pattern of 4E-BP1 expression is seen in PanINs from pancreas of mice that carry a pancreas-specific mutated Kras allele (Kras-Pdx1/cre mice). Furthermore, we show in vitro that 4E-BP1 expression is efficiently induced under hypoxia in Smad4^{+/+}, but not in Smad4^{-/-} pancreatic duct cells. We further reveal that, at the molecular level, Smad4 cooperates with HIF1- α to induce 4E-BP1 gene transcription in hypoxic cells. Thus, it appears that pancreatic duct cells which carry Smad4 loss are no longer capable of inducing 4E-BP1 under stress conditions. This may have important consequences in pancreatic carcinogenesis as 4E-BP1 is viewed as a stress-inducible factor whose function is to block cellular protein synthesis to concentrate resources on an appropriate stress response aimed at restraining carcinogenesis.

#3150 The SCF^{Fbw7} E3 ubiquitin ligase complex targets KLF5 for ubiquitination and degradation in a phosphorylation dependent manner. Han-Qiu Zheng, Dong Zhao, Zhongmei Zhou, Ceshi Chen. *Albany Medical College, Albany, NY.*

The KLF5 transcription factor is overexpressed in ER α negative basal type breast cancer and promotes breast cell proliferation, survival, and tumorigenesis through upregulating its target gene FGF-BP. We previously identified WWP1 as an E3 ubiquitin ligase for KLF5. However, the regulation mechanisms of KLF5 protein degradation through the ubiquitin proteasome pathway are still poorly understood. In this study, we demonstrate that the Skp1-Cul1-Fbw7 E3 ubiquitin ligase complex (SCF^{Fbw7}) targets KLF5 for ubiquitin-mediated degradation in a KLF5 phosphorylation dependent manner. Fbw7 interacts with KLF5 through the WD40 repeats of Fbw7 and a cdc-4 phospho-degron (CPD domain, ³⁰³SPPSS) of KLF5 and promotes KLF5 ubiquitination and degradation. Both the F-box and WD40 repeats of Fbw7 are required for KLF5 ubiquitination and degradation. Mutation of the critical Ser303 residue in the KLF5 CPD motif into Ala abolishes the protein interaction, ubiquitination, and degradation by Fbw7. In addition, the interaction between wild type KLF5 and Fbw7 can be abrogated by alkaline phosphatase (CIP) treatment. Using a KLF5 S303 phosphorylation specific antibody, we demonstrate that the S303 residue is phosphorylated. We further demonstrate that the KLF5 S303 can be phosphorylated by GSK3 in vitro. Inhibition of GSK3 activity by LiCl delays the KLF5 S303 phosphorylation and the KLF5 protein degradation. This new regulatory mechanism of KLF5 degradation may result in useful diagnostic and therapeutic targets for breast cancer and other cancers.

#3151 Posttranscriptional regulation of p21 by MCG10, an RNA-binding protein and a target of tumor suppressor p53. Ariane Scoumanne, Seong Jun Cho, Xinbin Chen. *Center for Comparative Oncology, University of California at Davis, Davis, CA.*

The tumor suppressor p53 plays a crucial role in regulating many cellular processes, such as cell cycle arrest, apoptosis, senescence and cell metabolism, in response to stress signals. As a transcription factor, p53 modulates the expression of a plethora of genes, including p21, Killer/DR5, GADD45, TIGAR and MDM2. The tumor suppressor function of p53 is further underscored by the high propensity of p53 mutations found in human cancer. RNA-binding proteins are key modulators in RNA metabolism involved in alternative splicing, mRNA stability, mRNA transport as well as translation. Abnormal expression of RNA-binding proteins is implicated in a growing number of human diseases ranging from neurological disorders to cancer. Here, we showed that MCG10, an RNA-binding protein and a target of p53, regulates the stability of cell cycle inhibitor p21 transcript. Indeed, we found that MCG10 decreases p21 levels induced upon DNA damage. In addition, we showed that MCG10 directly binds to the 3' untranslated region of p21 transcript to destabilize it. Furthermore, we generated MCF7 breast adenocarcinoma and RKO colon carcinoma cell lines in which MCG10 is inducibly knocked down. We found that MCG10 is required for cell proliferation. In addition, we showed that deficiency in MCG10 leads to an increase in basal and

DNA damage-induced p21 levels. Taken together, we uncovered a role for RNA-binding protein MCG10 in modulating the p53 pathway through posttranscriptional regulation of p21.

#3152 Sumoylation of eIF4E activates mRNA translation. Jing Hu, Xiang Xu, Jaya Vatsyayan, Chenxi Gao. *Univ. of Pittsburgh Cancer Inst., Pittsburgh, PA.*

Eukaryotic translation initiation factor 4E (eIF4E) is the cap-binding protein that binds the 5' cap structure of cellular mRNAs. Despite the obligatory role of eIF4E in cap-dependent mRNA translation, how the translation activity of eIF4E is controlled remains largely undefined. Here we report that mammalian eIF4E is regulated by SUMO-1 (small ubiquitin-related modifier-1) conjugation. eIF4E is sumoylated on lysines 36, 49, 162, 206 and 212. Using eIF4E sumo-deficient mutant as a tool, we find that eIF4E sumoylation disrupts the interaction of eIF4E with its inhibitor 4E-BP1 (eIF4E-binding protein 1) and promotes the eIF4E/eIF4G association. Further, downregulation of eIF4E sumoylation inhibits eIF4E-dependent protein translation of a subset of genes that are critical for cell proliferation and preventing apoptosis, and abrogates the oncogenic and anti-apoptotic functions associated with eIF4E. These data indicates that sumoylation represents a novel fundamental regulation mechanism of protein synthesis. Our findings further suggest that eIF4E sumoylation may be important in promoting human cancers.

#3153 The role of ubiquitin in androgen receptor function in prostate cancer. Steven Darby, Hollie Lumsden, Luke Gaughan, Craig N. Robson. *Newcastle Univ. Northern Institute for Cancer Research, Newcastle, United Kingdom.*

Introduction Prostate cancer (PC) is the most common male cancer in the western world. The androgen receptor (AR) plays a central role in PC development and remains the primary target for therapy. Androgen deprivation therapy is currently the standard treatment for PC. However, following a 2-3 year period, a more aggressive cancer usually recurs that is unresponsive to further androgen deprivation and is termed castrate resistant prostate cancer (CRPC). Several AR interacting proteins are components of the ubiquitin-proteasome degradation system, including Mdm2, CHIP and E6AP E3 ligases implicating proteasomal degradation/activation as a key event in AR function. In this study we have investigated which ubiquitin ligases are involved in AR ubiquitination and whether altered ubiquitination levels play a critical role and subsequent AR function in development of CRPC. Methods In vitro ubiquitination assays were performed with AR as the substrate with multiple purified E3 ligase enzymes in conjunction with wild type or mutant ubiquitin (K7R, K48 and K63). A panel of E2 conjugating enzymes were also employed to identify which E2/E3 heterodimers were involved in this process. Ubiquitinated AR fragments were gel excised and digested with trypsin. Fragments were then subjected to proteomic analysis to determine the sites of ubiquitination in AR. Results In vitro ubiquitination assays using lysine null ubiquitin (K7R) revealed monoubiquitination of AR in multiple domains of AR in response to four E3 ligases. Screening of an E2 panel also identified multiple E2 conjugating enzymes implicated in AR ubiquitination. K48 ubiquitin (implicated in proteasomal degradation) and K63 ubiquitin (implicated in substrate activation) incorporated into in vitro assays identified differential poly-ubiquitination chain patterns in AR, dependent on which E3 ligase was used in the assay. Proteomic analysis of ubiquitinated AR fragments identified a novel site of ubiquitination within AR. Conclusions AR was ubiquitinated by four E3 ligases evaluated in this study. Mono- and poly-ubiquitination of AR was identified. Mass spectroscopy revealed a novel site of ubiquitination in AR. Further experiments are being performed, including site directed mutagenesis to determine the importance of the novel ubiquitination site in AR protein proteasomal turnover and transcriptional activation.

#3154 Translation of TRAF1 is regulated by IRES-dependent mechanism and stimulated by vincristine in lymphoid malignancies. Lin Yang, Lubing Gu, Zhouya Li, Muxiang Zhou. *Emory Univ. School of Medicine, Atlanta, GA.*

TRAF1 is a member of the TRAF family, which plays important roles in signal transduction that mediate cell life and death in the immune response, inflammatory and malignant diseases. It is known that TRAF1 transcription is inducible by various cytokines, but little is known about the regulation of its protein translation. In the present study, we demonstrated that the human TRAF1 mRNA has an unusually long 5'-UTR that contains an IRES regulating TRAF1 protein translation. By performing gene transfection and reporter assays, we revealed that this IRES sequence is located within the 572 nucleotides upstream from the AUG start codon. An element between nucleotides -392 to -322 was essential for the IRES activity. Interestingly, we found that the TRAF1 expression is induced in cancer cells by chemotherapeutic drug vincristine that regulates cytoplasmic localization of PTB protein, which may contribute to the IRES-dependent translation of TRAF1 during vincristine treatment. These results indicate that TRAF1 translation is ini-

tiated via the IRES and regulated by vincristine, and suggest that regulation of the IRES-dependent translation of TRAF1 may be involved in effecting the cancer cell response to vincristine treatment.

#3155 The SCF^{FBW7} E3 ubiquitin ligase targets Krüppel-like factor 5 (KLF5) for proteolysis in breast cancer. Dong Zhao, Han-Qiu Zheng, Zhongmei Zhou, Ceshi Chen. *Albany Medical College, Albany, NY.*

Krüppel-like factor 5 (KLF5) is an evolutionarily conserved zinc finger-containing transcription factor promoting breast cell proliferation, survival, and tumor growth through upregulating its target gene FGF-BP. The protein abundance of KLF5 has been shown to be strictly regulated at transcriptional and posttranslational levels. Our previous studies suggest that the KLF5 protein degradation is through the ubiquitin proteasome pathway. The WWP1 E3 ligase can target KLF5 for ubiquitin-mediated proteasomal degradation. In this study, we demonstrated the F-box protein FBW7 targets KLF5 for proteolysis and suppresses KLF5's functions in breast cancer. The SCF^{FBW7} (SKP1-Cullin1-F-box) E3 ligase complex has been shown to target several oncoproteins, including MYC and CyclinE, for proteolysis. FBW7 could be a tumor suppressor due to its frequent mutations in multiple cancers. Here, we first demonstrated that inactivation of endogenous FBW7 by gene knockout, knockdown, or mutation remarkably increases the endogenous KLF5 protein abundances through blocking its degradation in multiple cell lines. Second, we found that overexpression of FBW7 decreases the exogenous and endogenous KLF5 protein levels in HEK293T and SUM149PT cell lines. Functionally, overexpression of FBW7 in the endogenous FBW7 mutated SUM149PT cells decreases the FGF-BP gene expression and the DNA synthesis. Importantly, knockdown of endogenous Fbw7 increases the FGF-BP gene expression, cell proliferation, and survival through upregulating the KLF5 expression in breast cells. These findings suggest that the Fbw7 tumor suppressor inhibit breast cancer cell proliferation and survival at least partially through targeting KLF5 for ubiquitin-mediated degradation. This new regulatory mechanism of KLF5 degradation may result in useful diagnostic and therapeutic targets for breast cancer and other cancers.

#3156 eIF4E in human breast cancer and its role in regulating translation of splice variants of breast cancer genes. Jenny Yuen-Nei Cheung, Ashley San-Yu Wong, Kelvin Yuen-Kwong Chan, Ui-Soon Khoo. *The University of Hong Kong, Hong Kong, Hong Kong.*

In eukaryotes, most of mRNAs are translated by the scanning mechanism. The trimeric complex eIF4F (consisting of eukaryotic initiation factor eIF4E, eIF4G and eIF4A) binds to ribosomes and the 5' end of mRNA, unwinding the secondary structures for scanning. eIF4E is the least abundant initiation factor, and may therefore determine the rate of translation. Overexpression of eIF4E can cause malignant transformation of immortalized human cells. Anti-sense oligonucleotides of eIF4E partially reversed the malignant phenotypes, suggesting that overexpression of eIF4E is the direct cause of transformation. Two- to threefold higher eIF4E levels have been detected in breast cancer using immunohistochemistry. About 10% of cellular mRNAs contain atypically long 5'UTR and many of these encode proto-oncogenes and growth factors. It is believed that these long 5'UTR sequences tend to form stable secondary structure, requiring higher levels or activities of eIF4F for their translation. Over 60% of human genes are alternatively spliced. These may produce variants with different degrees of secondary structure at their 5'-UTR. We hypothesized that the elevated eIF4E level in breast cancer cells allows more effective translation of breast cancer gene variants with extensive 5'UTR secondary structure. These changes in variants profile may be important for breast cancer development and progression. We first analyzed the expression levels of eIF4E in human breast cancer and non-tumor biopsies in tissue microarray. Our preliminary results showed that there was significantly higher eIF4E expression in tumor versus non-tumor and eIF4E expression was positively correlated to estrogen receptor (ER) expression (OR=4.4, p=0.006). Although eIF4E expression levels were independent of tumor grade and size, it was strongly associated with overall clinical ER and progesterone receptor status (OR=6.3, p=0.003 and OR=6.5, p=0.002). The differential translation of breast cancer gene splice variants in breast cancer cell lines is being analyzed and their correlation with the extent of secondary structure of their 5'UTRs will be determined.

#3157 Subcellular localization and mRNA targets of a novel human RNA-binding protein, KIAA0020. Ching-Wen Yang, Shu-Yu Cheng, Christina L. Chang. *National Cheng Kung University, Tainan, Taiwan.*

Human KIAA0020 belongs to the PUF family of RNA-binding proteins. Most of family members contain eight RNA-binding Pumilio repeats and are known to regulate mRNA stability and translation. In fact, hunchback, Cyclin B, and HO endonuclease mRNAs are known targets for certain PUF family members in human and other species. Currently little is known about human KIAA0020, which con-

tains six instead of eight Pumilio repeats and shares low homology with its family members. In this study, we characterized this protein and found that wild-type KIAA0020 was localized to multiple foci within the nucleus of HeLa and HEK293T cells. Deletion of C-terminus and, to a less extent, N-terminus of KIAA0020, permitted partial cytosolic localization. Furthermore, we have found a number of mRNA targets that were regulated by KIAA0020 in cells containing ectopic overexpression of KIAA0020 variants or shRNA-mediated knockdown. Based on microarray data analysis from Oncomine, KIAA0020 appears to be down-regulated in a variety of human cancer types. Understanding the subcellular localization and mRNA targets of KIAA0020 will improve our knowledge of the function of this protein and its relevance to cancer.

#3158 Evidence for a role of EWS in alternative splicing in Ewing's sarcoma. Lucia T. Riedmann, Maximilian O. Kauer, Gunhild Jug, Heinrich Kovacs. *Children's Cancer Research Institute, Vienna, Austria.*

Ewing's sarcoma family tumors (ESFT) are characterized in 85% of cases by the oncogenic fusion protein EWS-FLI1 resulting from a chromosomal translocation. While FLI1 is an ETS transcription factor, EWS belongs to the evolutionarily conserved FET family of RNA-binding proteins. So far, functional studies of oncogenic EWS fusion proteins have concentrated on their presumed role as transcriptional regulators. However, chimeric FET proteins are usually co-expressed with their intact counterparts and previous protein interaction studies have shown that EWS and EWS-FLI1 interact with each other. Therefore, it is possible that functional interference with normal EWS function accounts for part of the oncogenic activities of EWS-FLI1. However, the role of EWS in post-transcriptional and transcriptional gene regulation is poorly defined. Since FET proteins associate with a number of RNA processing factors, we investigated the influence of modulated EWS expression on genome wide splicing using Affymetrix HuEx-1.0stv2 arrays. For that purpose, we used two model systems: a unique ESFT cell line lacking endogenous EWS was studied upon stable restoration of EWS expression, and an ESFT cell line allowing for inducible RNAi-mediated EWS-FLI1 suppression was analyzed upon transient EWS knockdown. As a result, we found significant differences between cells with and without EWS expression in splicing patterns as well as alternative promoter usage for more than 50 genes. GO analysis of the top ranked genes is consistent with previously suggested functions of the FET protein family such as DNA repair, chromatin structure and RNA transcription. The alternative splicing patterns were verified by RT-qPCR of candidate genes which are currently tested for their functional relevance to ESFT. One of these candidate genes, structural maintenance of chromosome 5 (SMC5). As a core component of the SMC5-SMC6 complex it is involved in sister chromatid homologous recombination by recruiting the SMC1-SMC3 cohesin complex to DNA double-strand breaks during anaphase. Ongoing studies concentrate on the influence of EWS-FLI1 on EWS-dependent alternative RNA processing patterns in these model systems. This study is supported by grant P20665-B12 of the Austrian Science Fund FWF.

#3159 RPS6 controls the translation of 5' terminal oligopyrimidine tract-containing mRNAs through a physical interaction that regulates the loading of these messages on the polysome. Patrick Hagner, Krystyna Mazan-Mamczarz, Bojie Dai, Ronald B. Gartenhaus. *Univ. of Maryland, Baltimore, MD.*

The prevailing paradigm for mTOR signaling implicates RPS6 activation, translational control of mRNAs characterized by the presence of an oligopyrimidine tract (5' terminal oligopyrimidine) within the 5' untranslated region. These messages containing an oligopyrimidine tract are important for ribosomal biogenesis as they encode most of the translational apparatus. However, mechanistic details how RPS6 is involved in the regulation of these oligopyrimidine tract-containing messages are still poorly understood. We demonstrated that through small inhibitory RNA (siRNA) knockdown of RPS6, 5' TOP mRNA were selectively upregulated in heavy polysomal fractions. The ability of RPS6 to interact with endogenous mRNA containing 5' TOP sequences was tested by immunoprecipitation (IP) analysis using lysates from Diffuse Large B-cell Lymphoma (DLBCL) cell line (Farage and OCI-LY10) and a specific RPS6 antibody. The association of RPS6 with 5' TOP mRNA was interrogated using RT-qPCR to examine mRNAs isolated from the IP material using primer sets specific for multiple 5' TOP mRNAs. We found that 5' TOP containing mRNA were highly enriched in IP material obtained with anti-RPS6 antibody compared with the background level of amplification seen in control immunoglobulin G (IgG) IP. We constructed a heterologous GFP fluorescent protein (GFP) mRNA containing the 5' TOP sequence from the RPS6 gene in the 5' UTR. Using mRNP-IP assays, RPS6 was found to interact with a heterologous construct containing the 5' TOP sequence when compared to a normal GFP mRNA control. A number of previous publications indicated that aberrant control of protein translation contributes to lymphomagenesis. RPS6 has been shown to control the formation of nascent ribosomal subunits and to part-

The WWP1 E3 Ubiquitin Ligase as a Novel Molecular Target in Breast Cancer

Dr. Ceshi Chen

Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York

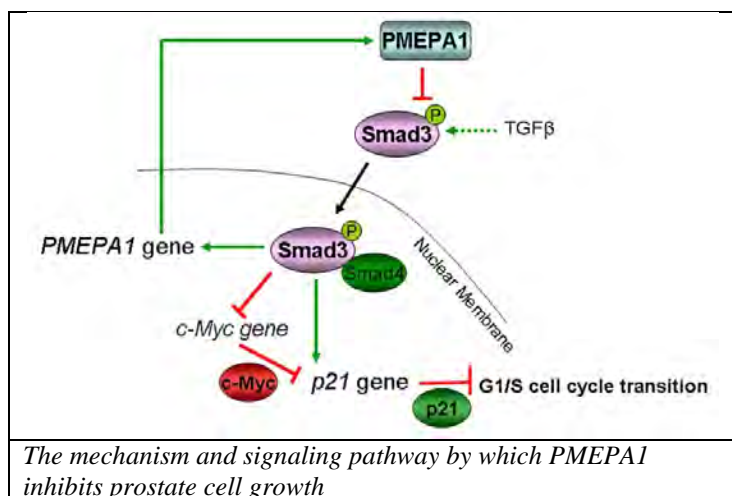
Protein ubiquitination is an important posttranslational modification that regulates a multitude of cancer related cellular processes, including cell growth and death. Protein ubiquitination is typically sequentially mediated by three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). The E3 controls substrate specificity. In human beings, there are more than 600 E3s. Interestingly, many E3s undergo frequent genetic and expression aberrations and function as either tumor suppressors (i.e., BRCA1) or oncoproteins (i.e., Mdm2 and Skp2) in breast cancer. Several specific and effective small molecular inhibitors of Mdm2 have been reported to have a substantial p53-dependent anti-tumor effect *in vivo*. Therefore, oncogenic E3s are promising molecules for cancer target therapy. Our long term goal is to identify cancer related E3s as molecular targets and to improve cancer prevention, diagnosis, and therapy. We have recently demonstrated that the WW domain containing E3 ubiquitin protein ligase 1 (WWP1) gene is frequently amplified and overexpressed in estrogen receptor α (ER α) positive breast cancer. Inhibition of WWP1 alone by siRNA or dominant negative WWP1 induces apoptosis in ER α positive breast cancer cell lines MCF7 and HCC1500 primarily through activation of caspase 8. The WWP1 siRNA induced apoptosis in MCF7 can be completely rescued by overexpression of wild type WWP1 but not the E3 ligase inactive WWP1C890A. Furthermore, we demonstrated that inhibition of WWP1 can be combined with tamoxifen or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) to suppress breast cancer cell proliferation and survival. Finally, we demonstrated that WWP1 may promote breast cancer cell survival partially through directly targeting several substrates, including p63 and ErbB4, for ubiquitin-mediated degradation. These findings suggest that WWP1 may be a novel molecular target in ER α positive breast cancer.

Poster P5-1

PEMPA1 PROMOTES PROSTATE CELL PROLIFERATION THROUGH SUPPRESSING THE SMADS-C-MYC-P21 SIGNALING PATHWAY

Ceshi Chen,¹ Rong Liu,¹ and Jian Huang²

¹Albany Medical College and ²Texas Medical Center



Background: The *PMPA1* gene has been shown to be an androgen-induced gene predominantly expressed in prostate epithelial cells and overexpressed in androgen-independent xenograft tumors. Transforming growth factor β (TGF β) has been shown to induce the *PMPA1* expression. Interestingly, PMPA1 suppresses the TGF β signaling through binding to Smad2/3 and preventing them from phosphorylation. However, PMPA1 has also been shown to have tumor suppressor activities. Thus, the physiological function and mechanistic action of PMPA1 in prostate cancer are unclear.

Methods: The expression of PMPA1 in prostate cancer was detected by western blotting (WB) and immunohistochemistry (IHC). PMPA1 was knocked down and overexpressed in PC-3 and other cancer cell lines. Cell proliferation was measured by DNA synthesis. Cell cycle was measured by propidium iodide staining and flow cytometry. The gene/protein expression levels were assessed by qRT-PCR and WB. Tumor growth was analyzed in nude mice.

Results: We demonstrate that PMPA1 is frequently overexpressed in prostate cancer cell lines and tumors. In RWPE1 and PC-3 prostate cell lines, inhibition of PMPA1 suppresses cell proliferation through upregulating the *p21* transcription. Additionally, PMPA1 overexpression suppresses the *p21* expression and promotes cell proliferation. We further found that PMPA1 is induced by TGF β as a negative feedback loop to suppress Smad3 phosphorylation and nuclear translocation, upregulates c-Myc, downregulates *p21*, and promotes cell proliferation. The PMPA1 functions depend on its Smad2/3 binding motif. Consistently, depletion of Smad3/4, but not Smad2, blocks the PMPA1's functions of regulating c-Myc and *p21*. Finally, stable depletion of PMPA1 in PC-3 inhibits xenograft growth.

Conclusions: PMPA1 promotes prostate cancer cell proliferation and tumor growth through inhibiting the Smad3/4-c-Myc-*p21* signaling pathway.

Impact: This study clarified the role of PMPA1 and mechanism of PMPA1 action in prostate cancer. PMPA1 could be a prognostic biomarker and therapeutic target in prostate cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0191.

MONDAY PM (Short Talk)

The Fbw7 tumor suppressor targets KLF5 for ubiquitin mediated degradation in a phosphorylation dependent manner

Ceshi Chen, Dong Zhao, Han-Qiu Zheng, Zhongmei Zhou

Albany Medical College, Albany, NY, USA

The KLF5 transcription factor is overexpressed in ER negative basal type breast cancer and promotes breast cell proliferation, survival, and tumorigenesis through upregulating its target gene FGF-BP. Our previous studies suggest that the KLF5 protein turns over rapidly by the WWP1 E3 ligase through the ubiquitin proteasome pathway. Recently, we demonstrate that the Skp1-Cul1-Fbw7 E3 ubiquitin ligase complex targets KLF5 for ubiquitin-mediated degradation in a KLF5 phosphorylation dependent manner. Fbw7 interacts with KLF5 through the WD40 repeats of Fbw7 and a CPD motif (303 SPPSS) of KLF5 and promotes KLF5 ubiquitination and degradation. Both the F-box and WD40 repeats of Fbw7 are required for KLF5 ubiquitination and degradation. Mutation of the critical S303 residue in the KLF5 CPD motif abolishes the protein interaction, ubiquitination, and degradation by Fbw7. Using a KLF5 phosphorylation specific antibody and the *in vitro* kinase assay, we demonstrate that the KLF5 S303 can be phosphorylated by GSK3 β . Inhibition of GSK3 β activity by LiCl delays the KLF5 degradation. Importantly, inactivation of endogenous Fbw7 by gene knockout, knockdown, or mutation remarkably increases the endogenous KLF5 protein abundances through blocking its degradation. The endogenous Fbw7 isoforms can promote endogenous KLF5 degradation in a cell line dependent manner. Finally, we found that endogenous Fbw7 suppresses the FGF-BP gene expression and breast cell proliferation through targeting KLF5 for degradation. These findings suggest that the Fbw7 tumor suppressor inhibit breast cancer cell proliferation at least partially through targeting KLF5 for ubiquitin-mediated degradation. This new regulatory mechanism of KLF5 degradation may result in useful diagnostic and therapeutic targets for breast cancer and other diseases.

The research is supported by an American Cancer Society Research Scholar Award and a DoD IDEA Award.

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Presentation Abstract

Abstract Number: LB-137

Presentation Title: **YAP promotes breast cell proliferation and survival partially through stabilizing the KLF5 transcription factor**

Location: Exhibit Hall A4-C, Poster Section 39

Author Block: Xu Zhi, Dong Zhao, Zhongmei Zhou, Rong Liu, Ceshi Chen. Albany Medical Center, Albany, NY

Abstract Body: The Yes-associated protein (YAP) is an oncoprotein in the Hippo tumor suppressor pathway that regulates tumorigenesis. Although YAP functions through its WW domains, the YAP WW domain binding partners have not been completely determined. In this study, we demonstrate that YAP functions partially through binding to KLF5, a transcription factor promoting breast cell proliferation and survival. YAP interacts with the KLF5 PY motif through its WW domains, prevents WWP1, a KLF5 E3 ubiquitin ligase, from binding to KLF5, and stabilizes KLF5 by decreasing the WWP1-mediated KLF5 ubiquitination and degradation. Over-expression of the wild type but not WW domain deficient YAP up-regulates the KLF5 protein levels and the mRNA expression levels of the KLF5 downstream target genes, including *FGF-BP* and *ITGB2*. In addition, knockdown of YAP decreases the expression levels of KLF5, FGF-BP, and ITGB2. Depletion of either YAP or KLF5 decreases breast cell proliferation and survival in MCF10A and SW527 breast cells. Importantly, stable knockdown of YAP or KLF5 significantly suppresses SW527 xenograft growth in mice. The YAP upstream kinase LATS1 suppresses the KLF5-FGF-BP signaling axis and cell growth through YAP. Finally, both YAP and KLF5 are co-expressed in estrogen receptor (ER α)-negative breast cell lines. These findings suggest that KLF5 is an important transcription factor partner for YAP and contributes to the Hippo tumor suppressor pathway. This study is supported by a grant from the American Cancer Society (RSG-08-199-01) and a grant from the Department of Defense (W81XWH-07-1-0191).

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Presentation Abstract

Abstract
Number: 4544

Presentation
Title: The induction of KLF5 transcription factor by progesterone contributes to progesterone-induced breast cancer cell proliferation and de-differentiation

Presentation
Time: Tuesday, Apr 05, 2011, 1:00 PM - 5:00 PM

Location: Exhibit Hall A4-C, Poster Section 29

Poster
Section: 29

Poster Board
Number: 13

Author
Block: Rong Liu, Dong Zhao, Zhongmei Zhou, Ceshi Chen. Albany Medical College, Albany, NY

Abstract
Body: Progesterone (Pg) promotes normal breast development during pregnancy and lactation and increases the risk of developing basal-type invasive breast cancer. However, the mechanism of action of Pg has not been fully understood. In this study, we demonstrate that the mRNA and protein expression of KLF5, a pro-proliferation and pro-survival transcription factor in breast cancer, was dramatically up-regulated in mouse pregnant and lactating mammary glands. Pg, but not estrogen and prolactin, induced the expression of KLF5 in multiple Pg receptor (PR)-positive breast cancer cell lines. Pg induced the KLF5 transcription is through PR in the PR-positive T47D breast cancer cells. Importantly, Pg failed to promote T47D cell proliferation when the KLF5 induction was blocked by siRNA. In addition, KLF5 overexpression was sufficient to induce the cytokeratin 5 (CK5) expression and the induction of CK5 by Pg was partially abrogated by KLF5 siRNA. Consistently, the expression of KLF5 was positively correlated with that of CK5 in a panel of breast cancer cell lines. Taken together, we conclude that KLF5 is a Pg-induced gene that contributes to Pg-mediated breast epithelial cell proliferation and de-differentiation. This work was supported by a grant from the American Cancer Society (RSG-08-199-01) and a grant from the Department of Defense (W81XWH-07-1-0191).

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Presentation Abstract

Abstract Number: LB-138

Presentation Title: **TAZ antagonizes the WWP1-mediated KLF5 degradation and promotes breast cell proliferation**

Location: Exhibit Hall A4-C, Poster Section 39

Author Block: Dong Zhao, Xu Zhi, Zhongmei Zhou, Ceshi Chen. Albany Medical College, Albany, NY

Abstract Body: Krüppel-like factor 5 (KLF5) is a PY motif-containing transcription factor promoting breast cell proliferation. The KLF5 protein is rapidly degraded through the proteasome after ubiquitinated by E3 ubiquitin ligases, such as WWP1 and SCF^{Fbw7}. In this study, we demonstrate that transcriptional co-activator with PDZ binding motif (TAZ) upregulated KLF5 through antagonizing the WWP1-mediated KLF5 ubiquitination and degradation. TAZ interacted with KLF5 through the WW domain and the PY motif, which is the binding site of WWP1. TAZ inhibited WWP1-mediated KLF5 ubiquitination and degradation. Overexpression of TAZ up-regulated the protein levels of KLF5 and FGF-BP, which is a well established KLF5 target gene. In addition, depletion of TAZ in both 184A1 and HCC1937 breast cells dramatically down-regulated protein levels of KLF5 and FGF-BP and inhibited cell growth. Furthermore, stable depletion of either TAZ or KLF5 significantly suppressed HCC1937 xenograft growth in SCID mice. Knockdown of LATS1, a TAZ upstream inhibitory kinase, up-regulated the protein levels of KLF5 and FGF-BP in 184A1 and promoted cell proliferation through TAZ. Finally, both KLF5 and TAZ are co-expressed in a subset of ER-negative breast cell lines. These results, for the first time, suggest that TAZ promotes breast cell growth partially through protecting KLF5 from WWP1-mediated degradation, and enhancing KLF5's activities. This study is supported by a grant from the American Cancer Society (RSG-08-199-01) and a grant from the Department of Defense (W81XWH-07-1-0191).

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Presentation Abstract

Abstract
Number: 1957

Presentation
Title: PMEPA1 promotes androgen receptor negative prostate cell proliferation through suppressing the Smad3/4-c-Myc-p21^{Cip1} signaling pathway

Presentation
Time: Monday, Apr 04, 2011, 1:00 PM - 5:00 PM

Location: Exhibit Hall A4-C, Poster Section 1

Poster
Section: 1

Poster Board
Number: 27

Author
Block: Rong Liu¹, Zhongmei Zhou¹, Jian Huang², Ceshi Chen¹. ¹Albany Medical College, Albany, NY; ²Baylor College of Medicine, Houston, TX

Abstract
Body: The *PMEPA1* gene has been shown to suppress the androgen receptor (AR) and TGF β signaling pathways and is abnormally expressed in prostate tumors. However, the role and mechanism action of PMEPA1 in AR-negative prostate cancer are unclear. Here, we demonstrate that inhibition of PMEPA1 suppresses AR-negative RWPE1 and PC-3 prostate cell proliferation through upregulating the *p21* transcription. Additionally, PMEPA1 overexpression suppresses the p21 expression and promotes cell proliferation. PMEPA1 is induced by TGF β as a negative feedback loop to suppress Smad3 phosphorylation and nuclear translocation, upregulates c-Myc, downregulates p21, and promotes PC-3 cell proliferation. The PMEPA1 functions depend on its Smad2/3 binding motif. Consistently, depletion of Smad3/4, but not Smad2, blocks the PMEPA1's functions of regulating c-Myc and p21. Importantly, stable depletion of PMEPA1 in PC-3 inhibits xenograft growth. Finally, we found that PMEPA1 is overexpressed in a subset of prostate cancer cell lines and tumors. These findings suggest that PMEPA1 may promote AR-negative prostate cancer cell proliferation through p21. (This work was supported by a grant from the Department of Defense (W81XWH-07-1-0191) and a grant from the American Cancer Society (RSG-08-199-01). Dr. Liu is supported by a postdoctoral fellowship from American Urological Association Foundation.)

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